Apoptosis induced by β-amyloid$_{25-35}$ in acetylcholinesterase-overexpressing neuroblastoma cells[1]

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ABSTRACT

AIM: To examine the relationship between apoptosis induced by β-amyloid fragment 25-35 (Aβ$_{25-35}$) and the activity of acetylcholinesterase (AChE) in AChE over-expresser—SC42 cells. **METHODS:** Cell survival was measured by microscopy and MTT reduction; DNA laddering was observed by electrophoresis; AChE activity was determined by spectrophotometry. **RESULTS:** Aβ$_{25-35}$ 1 μmol/L exposure for 24-48 h caused a significant decrease in cell viability, along with changes in morphology and DNA fragmentation. AChE activity was affected in an inverse manner, increasing gradually to a level that was 1.7-fold higher than control at the 48-h time point. No change in the cytotoxicity of Aβ$_{25-35}$ was observed when the increased AChE activities were effectively inhibited by huperzine A throughout the 48-h exposure period. **CONCLUSION**: Although Aβ$_{25-35}$ can induce apoptosis in SC42 cells and simultaneously increase AChE activity, the capacity of AChE to hydrolyze acetylcholine is not involved in this apoptosis model.

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia in adults, is a neurodegenerative disorder characterized by selective neuronal loss and the presence of two different types of fibril deposits: senile plaques and neurofibrillary tangles[1]. The core of senile plaques is mainly composed of an amyloid β-peptide (Aβ) of 40-43 amino acids, which is neurotoxic when it forms amyloid fibrils *in vitro*[2] and *in vivo*[3]. The occurrence of neurodegenerative changes around amyloid plaques, and the dependence of Aβ-neurotoxicity on the formation of amyloid fibrils, indicates that amyloid aggregation is the primary pathogenic mechanism in AD[2]. However, the precise mechanism by which Aβ induces neuronal cell death, including apoptosis, remains unclear. It has recently been suggested that endogenous factors such as acetylcholinesterase (AChE) play a significant role in modulating the cytotoxicity of Aβ[4]. In the AD brain, AChE is associated predominantly with the amyloid core of mature senile plaques, diffuse "pre-amyloid" deposits, and the endothelial lining cerebral blood vessels[5]. More relevant, the brain areas where senile plaques are present are strongly AChE positive[6]. AChE also directly promotes assembly of Aβ peptide into amyloid fibrils, whose toxicity is higher than that of the Aβ aggregates alone[4]. Since such findings have suggested that there is close interaction between AChE and Aβ, it has become of interest to know the role of AChE activity in Aβ-

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induced apoptosis. Here, we employed cell lines that over-express or underexpress AChE[7], together with huperzine A (HupA), a selective acetylcholinesterase inhibitor isolated from Chinese herb[8], to explore the relationship between AChE activity and Aβ-caused apoptosis.

MATERIALS AND METHODS

Materials AChE over-expressing SC42 cells and under-expressing SC35 cells were established in the Department of Molecular Pharmacology, Mayo Clinic. The cells were stable transfectants of the N1E 15 neuroblastoma line, incorporating a plasmid containing full-length cDNA for murine AChE (sense and antisense orientation, respectively) under the control of a beta-actin promoter[7]. HupA, a colorless powder with purity >98 %, was prepared by Department of Phytochemistry, Shanghai Institute of Materia Medica, and was dissolved with distilled water. G418 and Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), benzylpenicillin 100 kU/L, and streptomycin 100 mg/L were from Gibco. Sodium dodecyl sulfate (SDS), and Coomassie brilliant blue (G250) were purchased from Fluka Chemie. Other reagents were from Sigma Chemical: 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl-tetrazolium bromide (MTT), β-amyloid peptide25-35 ($A\beta_{25-35}$), acetylthiocholine iodide (s-ACh), 5,5´-dithio-*bis* (2-nitrobenzoic acid) (DTNB), and tetraisopropyl pyrophosphoramide (isoOMPA). All chemicals were of reagent grade.

Cell cultures and $A\beta_{25-35}$ exposure Cells were seeded into multiwell plates at a density of 5×10^7 cells/L in DMEM with 0.5 g/L G418 and were maintained at 37 ºC in a humidified atmosphere containing 5 % CO_2. Experiments were carried out 24-48 h after seeding. After pretreating cultures with HupA for 2 h (0.1-10 μmol/L), $A\beta_{25-35}$ was added (1 μmol/L final concentration). Assays for cell survival, DNA fragmentation, and AChE activity were performed 24 and 48 h after $A\beta_{25-35}$ exposure. Cell survival was evaluated by two different methods: 1) morphological observations with a phase-contrast microscope (Nikon) and 2) measurements of the ability to reduce MTT, an indication of metabolic activity and mitochondrial integrity.

DNA fragmentation DNA fragmentation was analyzed by electrophoresis: briefly, cells were disrupted in 500 μL lysis buffer at 37 ºC overnight. DNA in the lysate was extracted with equal volumes of chloroform/isoamyl alcohol, then with chloroform. DNA was precipitated with 2 volumes of ethanol in the presence of 0.3 mol/L sodium acetate. After centrifugation at 12 000×*g* for 15 min, the DNA pellets were washed with 70 % ethanol, air-dried, and dissolved in 20 μL TE buffer (Tris-HCl 10 mmol/L, edetic acid 1 mmol/L, pH 7.6). The sample was electrophoresed on a 1.5 % agarose gel at 85 V for 1 h and photographed using an ultraviolet gel documentation system.

AChE activity assay The cultures were washed with ice cold PBS, pooled in a solution of 0.5 % Triton and 0.1 mol/L PBS buffered with edetic acid 0.05 mmol/L (pH 7.4), and then homogenized. Homogenates were centrifuged at 3000×*g*, 4 ºC for 20 min. The supernatants were used for determination of AChE activity by a standard spectrophotometric method[8].

Protein assay Protein concentration was measured by the Coomassie blue protein-binding method[9] using bovine serum albumin as standard.

Statistical analysis Data were expressed as % of control±SEM and were evaluated for statistical significance with one way ANOVA followed by Duncan's multiple range test.

RESULTS

Cell survival It was qualitatively apparent, as illustrated by the representative views in Fig 1, that an $A\beta_{25-35}$ 1 μmol/L insult caused SC42 cells to exhibit signs of degeneration, including pyknosis as well as blebbing and dissolution of neuritic processes. The cellular injury was aggravated with increasing incubation time. Thus, at the 48-h point, most cells demonstrated a rounded shape and were found floating in the medium; some were even lysed. Meanwhile, cell viability as determined by MTT reduction decreased progressively after $A\beta_{25-35}$ treatment, from 73 percent of control at 24 h, to 45 percent of control at 48 h ($P<0.01$, Fig 2).

DNA laddering To determine whether the $A\beta_{25-35}$ induced decrease in cell viability reflected death via apoptosis, a DNA fragmentation assay was used to detect DNA multimers of 180-240 bp, typically associated with endonuclease activity. In our experiment, genomic DNA exhibited a ladder pattern on electrophoresis after $A\beta_{25-35}$ exposure. DNA fragmentation was already apparent at the 24-h time point and it reached a maximum at 48 h (Fig 3).

AChE expression Measurements of cellular AChE activity after $A\beta_{25-35}$ treatment revealed changes that were dramatically opposite to the alteration in cell survival.

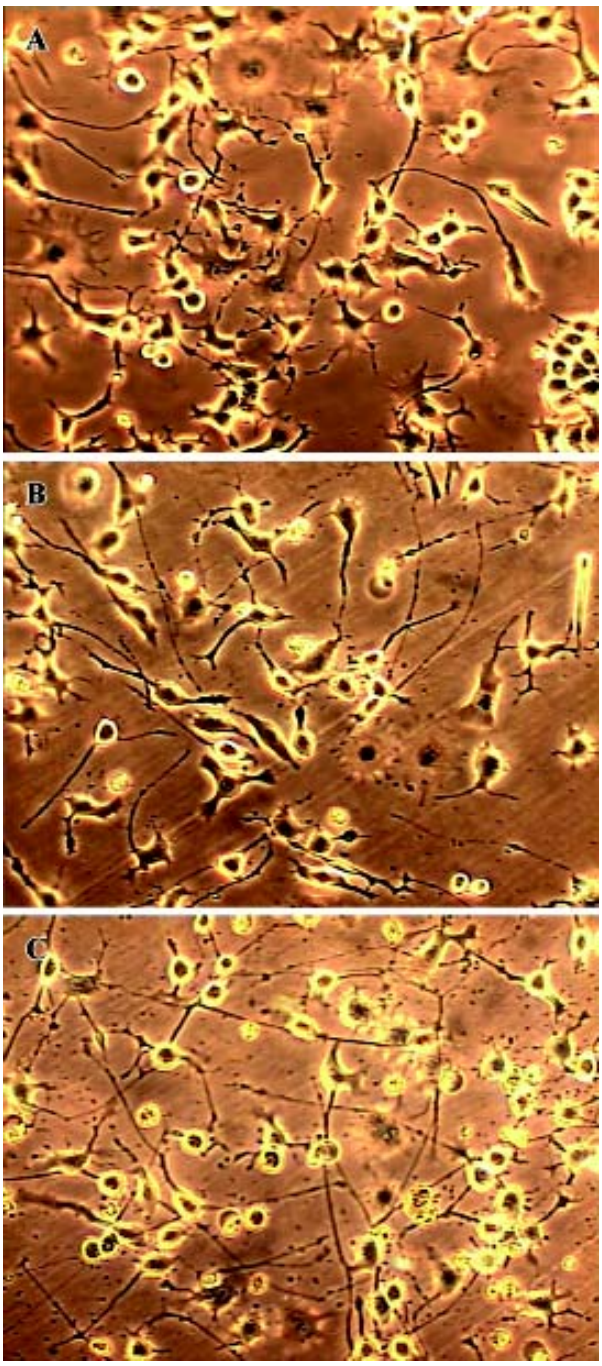

Fig 1. $A\beta_{25-35}$ induced injury in SC42 cells. Cultures were treated with vehicle (A), with $A\beta_{25-35}$ 1 μmol/L for 24 h (B), 48 h (C). Cell morphology was observed by phase-contrast microscopy (200×magnification).

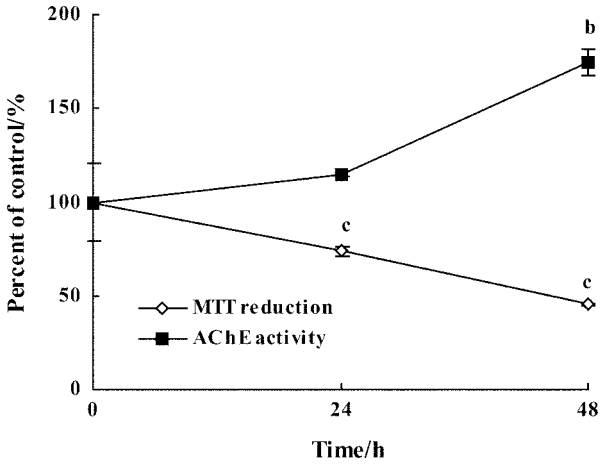

Fig 2. Changes of cell viability and AChE activity after $A\beta_{25-35}$ treatment. Cell viability was assessed by MTT-reduction assay. The concentration of $A\beta_{25-35}$ is 1 μmol/L. *n*=3. Mean±SEM. AChE activity of vehicle control was (3.6±0.7) OD_{440} values per mg protein. $^bP<0.05$, $^cP<0.01$ *vs* vehicle-treated cultures only.

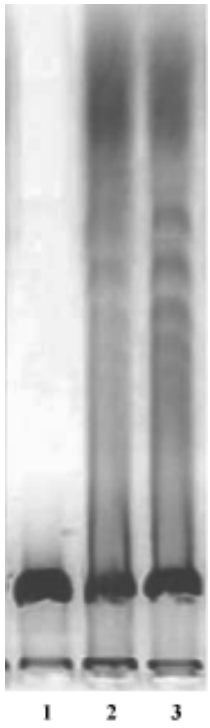

Fig 3. Reduction of $A\beta_{25-35}$-induced DNA fragmentation in SC42 cells. DNA extraction and agarose gel electrophoresis were performed as described in materials and methods. Lane 1: vehicle treatment; Lanes 2, 3: $A\beta_{25-35}$ 1 μmol/L exposure for 24 and 48 h, respectively.

Thus, mean AChE activity in SC42 cells was markedly enhanced to 174 percent of control level after 48 h of $A\beta_{25-35}$ exposure ($P<0.05$, Fig 2). In view of this and previous evidence that Aβ may interact with AChE, two experiments were performed to evaluate the possibility that Aβ-cytotoxicity is somehow related to AChE expression. The first experiment was to compare Aβ toxicity in SC42 cells with the toxicity in SC35 cells, a related neuroblastoma line engineered for under-expres-

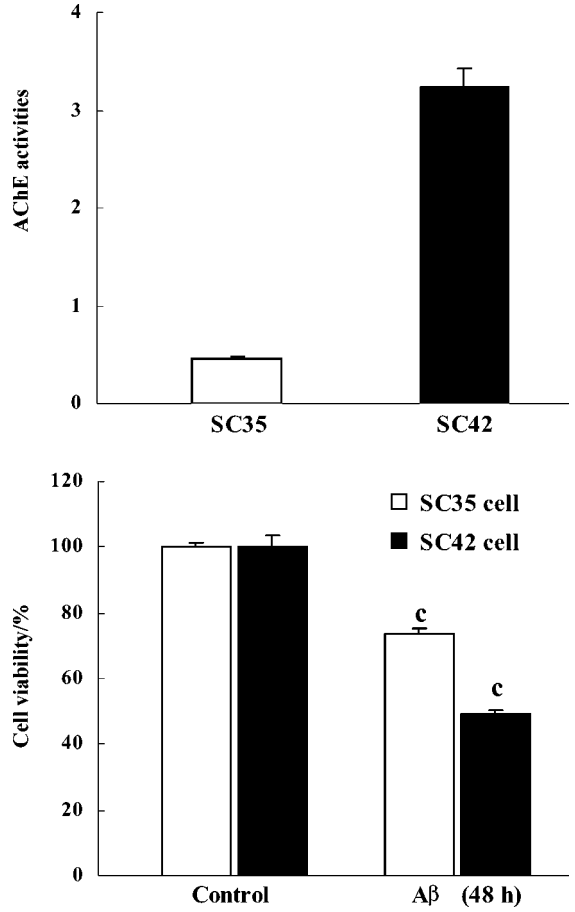

Fig 4. Comparative responses to $A\beta_{25-35}$ in cells expressing high and low AChE content. AChE-overexpressor (SC42) and underexpressor cells (SC35) were exposed to $A\beta_{25-35}$ 1 μmol/L for 48 h. Viability, assessed by MTT reduction, was compared with that of unexposed control cells from the respective lines. *n*=3. Mean±SEM. $^cP < 0.01$ *vs* vehicle-treated cultures. AChE activity was expressed as *OD* value per mg protein.

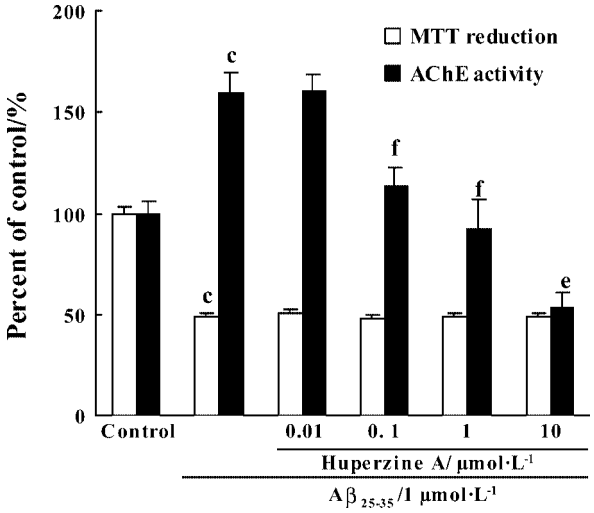

Fig 5. Effects of HupA on cell viabilities and AChE activities after $A\beta_{25-35}$ treatment. Exposure to HupA began 2 h before addition of $A\beta_{25-35}$ 1 μmol/L for 48 h. Cell viability was assessed by MTT-reduction assay. AChE activity was measured by the spectrophotometric method. *n*=3. Mean±SEM. AChE activity of vehicle control was (3.22± 0.19) OD_{440} values per mg protein. $^cP<0.01$ *vs* vehicle-treated cultures, $^eP<0.05$, $^fP<0.01$ *vs* $A\beta_{25-35}$-treated cultures only.

sion of AChE. As shown in Fig 4, these cells had 5-fold less amounts of AChE activity than SC42. It also became apparent that SC35 cells were much more resistant to the toxicity of Aβ-exposure, according to estimates of cell viability at 48 h (Fig 4).

In a further attempt to determine whether the increased AChE activity was involved in the decreased cell survival, Aβ was applied to SC42 cells that were pretreated with the selective AChE inhibitor, HupA (Fig 5). Pretreatment with HupA 0.1-10 μmol/L blocked the increase in AChE activity caused by 48-h Aβ-exposure ($P<0.01$, Fig 5). On the other hand, HupA had no effect on cell survival as measured by MTT reduction.

DISCUSSION

Programmed cell death or apoptotic cell death is regularly detected in the brain of AD patients[10] and apoptosis is thought to play an important role in the degeneration of neuronal tissue observed in AD[11]. Recently, a degradation product of the β-amyloid precursor protein (APP), referred to as Aβ , has been recognized as an invariable constituent of the characteristic neuronal plaques in AD[12]. It has also been shown that addition of purified Aβ and of its active fragment $A\beta_{25-35}$ can induce apoptosis in a variety of mammalian cell type *in vitro*[13,14]. Our finding that 1 μmol/L $A\beta_{25-35}$ treatment for 48 h can induce significant cytotoxicity and DNA fragmentation in SC42 cells is in agreement with available information.

The molecular mechanisms by which Aβ causes apoptosis are still a matter of debate. In view of the increasing evidence for close interactions between AChE and Aβ, and the possible role of AChE activity in Aβ-induced apoptosis, we compared two sub-lines of mouse neuroblastoma cells that, in principle, differed only in their ability to express AChE. This strategy allowed us to examine effects of AChE activity on $A\beta_{25-35}$-induced apoptosis. In the present study, we observed that AChE activity rose gradually with increasing time of exposure to Aβ, while cell viability decreased and DNA fragmentation became more apparent. The coincidence between the rise in AChE activity and the appearance of apoptosis

suggests that $A\beta_{25-35}$ exposure might directly cause both reactions. This result is in line with recent observations[15] which indicate that Aβ treatment causes increased expression of AChE by reducing the enzyme's rate of degradation. Our finding is also consistent with previous observations that, despite the overall depletion of AChE in the AD brain, there is elevated AChE content in the immediate vicinity of tangles and senile plaques[16]. One explanation for this finding may be that Aβ stimulates the expression of AChE in surrounding cells.

The greater loss of viability in SC42 cells than in SC35 cells during $A\beta_{25-35}$ exposure suggests that high AChE expression enhances vulnerability to $A\beta_{25-35}$ toxicity. It should be noted, however, that SC42 and SC35 cells differ, not only in AChE expression, but also in ability to differentiate and extend neurites. In fact we would argue that AChE expression in neuroblastoma is a key feature that promotes neural differentiation[7]. Thus, the greater vulnerability of SC42 to $A\beta_{25-35}$ may simply reflect the higher state of differentiation attained under our experimental conditions. That possibility is consistent with the results of our experiments with the selective AChE inhibitor, HupA.

Since pharmacologic blockade of AChE activity did not ameliorate the injury of SC42 cells exposed to $A\beta_{25-35}$, the catalytic function of AChE was probably not needed to promote $A\beta_{25-35}$-induced apoptosis. However, the AChE molecule may have other relevant functions. Beyond the active site, classically associated with acetylcholine hydrolysis, there is a peripheral anionic site (PAS), which can be important under certain conditions. For example there is a growing consensus that the PAS, or an adjacent area on the protein surface, is involved in neural adhesion and neurite extension[17,18]. Recent work suggests that this site could also be involved in AD pathogenesis. Thus, *in vitro* studies show that AChE will enhance the aggregation of $A\beta_{25-35}$ peptides by forming a stable complex with the growing fibrils[19]. Interestingly, the toxicity of such AChE-amyloid complexes is higher than that of the Aβ aggregates alone[4]. The interaction between AChE and Aβ is thought to involve a hydrophobic region on the enzyme surface, near the PAS[16]. In support of that idea, selective ligands for the PAS inhibit the ability of AChE to promote fibril formation by Aβ[20]. Additional work is needed to confirm this conclusion rigorously, using realistic *in vivo* models of AD, and to evaluate the full potential of AChE ligands as therapeutic agents for this disorder.

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