Limited activation of the intrinsic apoptotic pathway plays a main role in amyloid-β-induced apoptosis without eliciting the activation of the extrinsic apoptotic pathway

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Abstract. Amyloid-β (Aβ), a main pathogenic factor of Alzheimer's disease (AD), induces apoptosis accompanied by caspase activation. However, limited caspase activation and the suppression of the intrinsic apoptotic pathway (IAPW) are frequently observed upon Aβ treatment. In this study, we investigated whether these suppressive effects of Aβ can be overcome; we also examined the death-related pathways. Single treatments of cells with Aβ42 for up to 48 h barely induced caspase activation. In cells treated with Aβ42 twice for 2 h followed by 22 h (2+22 h) or for longer durations, the apoptotic protease activating factor-1 (Apaf-1) apoptosome was formed and caspases-3 and -9 were activated to a certain extent, suggesting the activation of the IAPW. However, the Aβ42-induced activation of the IAPW differed from that induced by treatment with other agents, such as staurosporine (STS) in that lower amounts of cytochrome *c* were released from the mitochondria, the majority of procaspase-9 in the Apaf-1 complex was not processed and caspase-3 was activated to a lesser extent in the peptide-treated cells. Thus, it seemed that the IAPW was not fully activated by Aβ42. The 30- and 41/43-kDa fragments derived from procaspase-8 were detected, which appear to be produced through the IAPW without death-inducing signaling-complex (DISC) formation, a key feature of the extrinsic apoptotic pathway (EAPW). Bid cleavage was observed only after caspase-3 activity reached its maximal levels, suggesting that the cleavage may contribute in a limited capacity to the amplification process of the IAPW in the Aβ-treated cells. Taken together, our data suggest that the IAPW, albeit functional only to a limited extent, plays a major role in Aβ42-induced apoptosis without the EAPW.

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; DISC, death-inducing signaling-complex; EAPW, extrinsic apoptotic pathway; IAPW, intrinsic apoptotic pathway; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SEC, size-exclusion column chromatography; STS, staurosporine

Key words: amyloid-β, apoptosis, intrinsic apoptotic pathway, caspase, apoptosome

Introduction

Amyloid-β (Aβ) are peptides of 36-43 amino acids in length, known to elicit neurodegenerative Alzheimer's disease (AD) (1). The peptides are generated by the proteolytic cleavage of the amyloid precursor protein by α-, β- and γ-secretases (2,3). Monomeric Aβ is prone to the change in its conformation to β-sheet-rich intermediate structures. The intermediates interact with each other to form multimeric aggregates, such as oligomers, protofibrils and fibrils (4-6). The aggregated Aβ peptides are progressively deposited in the brain parenchyma and cerebral blood vessels (7). However, the soluble Aβ oligomers and protofibrils have been found to be more toxic than the deposited fibrils, suggesting that the oligomeric aggregates would be the main factor for AD (4,8).

An essential role of apoptosis in eliciting Aβ cytotoxicity has been proposed, as caspases are activated in cells treated with the peptide (9-12). Caspase, a hallmark enzyme of apoptosis, is synthesized as a zymogen that is activated by the apoptotic signal. The signal of receptor-mediated apoptosis or the extrinsic apoptotic pathway (EAPW) leads to processing and catalytic activation of caspase-8 through the formation of the death-inducing signaling complex (DISC) with other proteins, such as Fas-associated protein with death domain (FADD) (13). On the other hand, chemically-induced apoptosis or the caspase-dependent intrinsic apoptoticpathway (IAPW) activates caspase-9, which is associated with the adaptor protein, apoptotic protease activating factor-1 (Apaf-1), dATP and cytochrome *c* released from the mitochondria to form muti-protein complexes known as apoptosomes (12-14). Activated caspases-8 and -9 then process effector caspases, including caspases-3, -6 and -7, which subsequently transduce the death

signal by cleaving other proteins (15,16). The EAPW and IAPW are the two major apoptotic processes.

The binding of Aβ to receptors, such as DR4, DR5 or p75 neurotrophin receptor can trigger the activation of the EAPW (13,17,18), while impaired autophagic degradation of the damaged mitochondria during aging (19) may lead to the the accumulation of Aβ in the mitochondrial membrane in neurons followed by the release of cytochrome *c* (19,20), which can trigger the activation of the caspase cascade of IAPW (19). On the other hand, caspase-4-mediated apoptosis is also induced by the unfolded protein response signaling pathway when endoplasmic reticulum stress is prolonged by the peptide (21). The differential activation of each pathway depends on the proteins or factors that interact with Aβ (22,23) and the conformational states of Aβ (oligomer vs. fibril) (12,24,25).

Although a number of studies have established a major role for apoptosis in Aβ-induced cell death, it is frequently observed that caspase activation is not potent in Aβ-treated cells (see Results section) (26). Furthermore, our recent findings (27) indicated that caspase activation and cell death induced by staurosporine (STS), commonly employed to induce the activation of the IAPW, are significantly reduced by Aβ42. The inhibitory effect of Aβ42 on the apoptotic pathway is associated with its interaction with procaspase-9 and the consequent inhibition of Apaf-1 apoptosome assembly. Similarly, it is also possible that Aβ42 interacts with other proteins involved in apoptosis and disrupts their functions, resulting in low levels of caspase activation. However, based on the studies regarding Aβ-induced apoptosis, we hypothesized that these inhibitory effect can be overcome in such a way that caspases can be activated. In the present study, we aimed to detect a condition under which the inhibitory effect of Aβ on apoptosis is overcome and caspases are activated, as well as to identify the apoptotic pathways involved in this condition.

Materials and methods

Materials. Fetal bovine serum (FBS) was purchased from Life Technology Inc. (Grand Island, NY, USA). Dulbecco's modified Eagle's medium, high glucose (DMEM/HG) was obtained from Welgene (Daegu, Korea). N-Acetyl-LEHD-amino methyl coumarin (Ac-LEHD-AMC), Ac-IETD-AMC, Ac-VEID-AMC and Ac-DEVD-AMC were from A.G. Scientific Inc. (San Diego, CA, USA). Anti-caspase-9 (cat. no. sc-7885), anti-caspase-3 (cat. no. sc-56053), anti-β-actin (cat. no. sc-47778) and goat anti-rabbit IgG-HRP (cat. no. sc-2030) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-caspase-8 antibody (cat. no. 9746) was from Cell Signaling Technology (Dedham, MA, USA). Anti-DFF45 antibody (cat. no. 611036) was from BD Transduction Laboratory (San Diego, CA, USA). Anti-caspase-6 (cat. no. YF-PA10680), anti-FADD (cat. no. YF-MA16512) and anti-cytochrome *c* (cat. no. LF-MA0182) antibodies were from AbFrontier (Seoul, Korea). Anti-Bid antibody was developed in laboratory. Recombinant mouse TNF-α (cat. no. 410-MT) was obtained from R&D Systems (Minneapolis, MN, USA) and actinomycin D (cat. no. A1410) from Sigma (St. Louis, MO, USA). All other chemicals were obtained from Sigma, unless otherwise stated.

Preparation of Aβ peptide. Aβ42 was purified from a fusion protein with GroES as previously described (28). After drying, the purified peptide powder was dissolved in 100% 1,1,1,3,3,3,-hexafluoro-2-propanol which was removed by evaporation under a fume hood and then under a vacuum. Nitrogen gas was added to the resulting Aβ42 powder and it was stored at -20˚C. Immediately before use, the peptide was dissolved in 0.1% NH4OH at a concentration of 2 mg/ml and then diluted at the desired concentration with the cell culture media without FBS. Aβ42 oligomers were prepared by dissolution of the peptide powder in cell culture media at 100 μM and incubation at 4˚C for 12 h as previously described (29). To prepare the fibrils, the Aβ42 powder at 100 μM was dissolved in phosphate-buffered saline (PBS) supplemented with 0.02% sodium azide and incubated at 37˚C for 4 days.

Cell culture and cell death assay. The human epithelial HeLa cells (30) were cultured in DMEM (HG) medium supplemented with 10% (v/v) FBS and 1% antibiotics (penicillin/streptomycin) at 37˚C under 5% CO_2. For cell death assay, the cells were seeded at a density of 15,000 cells/well in 96-well plates (Nunc, Roskilde, Denmark), cultured for 24 h, serum-deprived for a further 12 h and treated with Aβ42 for the indicated periods of time or STS (0.5 μM) for 6 h. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide formazan (MTT) reduction test (27), in which each well was supplemented with 20 μl of 5 mg/ml MTT solution in PBS, incubated for 2 h and then with 100 μl of a solubilization buffer [20% sodium dodecylsulfate (SDS) solution in 50% (v/v) DMF (pH 4.7)] for 12-16 h. For the assessment of cell viability, alamarBlue assay (31,32) was also employed, in which 10 μl of alamarBlue (Life Technologies, Carlsbad, CA, USA) were added directly to the cells and the mixture was incubated for 4-16 h. In the both assays, the absorbance was recorded at 570 nm using a microplate reader Spectra Max 190 (Molecular Devices, Sunnyvale, CA, USA).

Measurement of caspase activity. Caspase activity was measured as previously described (27). In brief, 20,000 cells/well were seeded in a 96-well plate, cultured for 24 h, and serum-starved for an additional 12 h. Following treatment with Aβ42 preparations or other damaging agents, the cells were washed twice with ice-cold PBS. Subsequently, 40 μl of lysis buffer (20 mM HEPES-NaOH, pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM EGTA, 20 mM NaCl, 0.25% Triton X-100, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin, 25 μg/ml N-acetyl-Leu-Leu-Norleu-al) were added to each well. The mixture was incubated on ice for 20 min. Caspase assay buffer (20 mM HEPES-NaOH, pH 7.0, 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 10 mM DTT) and substrates were then added, and the release of AMC was monitored for 2 h at 2-min intervals at excitation and emission wavelengths of 360 and 480 nm, respectively, using a microplate spectrofluorometer Gemini-XS (Molecular Devices). The results were expressed as the slope of total readings vs. time.

Western blot analysis. The cells were washed twice with ice-cold PBS and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM

EDTA, 5 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A and 2 μg/ml aprotinin) for 20 min on ice, as preivously desribed (27). The extracts were obtained by a microfuge centrifugation of the lysed cells at 13,000 rpm at 4˚C for 30 min. They were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins on the gel were transferred onto polyvinylidene difluoride membranes. The membranes were immunoprobed with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The blots were visualized using West-Zol plus reagent (Intron Biotechnology Inc., Seoul, Korea). Optical densities of immunoreactive bands were quantified using NIH ImageJ software (obtained in http://rsb.info.nih.gov/ij/), if necessary.

Analysis of cytochrome c release. The cells were washed twice with ice-cold PBS and resuspended in digitonin buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, and 190 μg/ml digitonin). After 5 min on ice, the supernatants and pellets were obtained by microfuge centrifugation at 14,000 rpm at 4˚C for 5 min. The supernatants were transferred to fresh tubes containing protease inhibitors (0.1 mM PMSF, 1 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin) and the pellets were resuspended in a buffer containing 25 mM Tris-HCl (pH 8.0) and 1% Triton X-100. 30 μg of protein from each sample was subjected to western blot analysis for the examination of the levels of cytochrome *c* as previously described (27).

Size exclusion column chromatography (SEC). The formation of the apoptosome and DISC were determined by SEC analysis of the cell extracts as previously described (33,34). For DISC formation study, cells were treated with 100 μg/ml of actinomycin D for 2 h followed by 50 nM of TNF-α for a further 24 h. The cell extracts were prepared as follows: The harvested cells were washed with ice-cold PBS twice and lysed in a lysis buffer containing 20 mM HEPES-KOH, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM KCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin and 25 μg/ml ALLN. The cell extracts were obtained by centrifuging the lysed cells at 13,000 rpm for 1 h at 4˚C. For SEC, cell extracts were loaded onto a Superose 6 HR (10/30) column (Amersham Pharmacia Biotech, Uppsala, Sweden) and 0.5 ml fractions were collected. Proteins in the fractions were concentrated using a 10-kDa cut-off membrane filter (Merck Millipore, Billerica, MA, USA) and analyzed by western blot analysis. The column was calibrated using calibration kits (Amersham Pharmacia Biotech) containing thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa).

Reconstitution of the cell-free apoptotic process. Cell-free apoptotic processes were induced by incubating cell extracts (70-1,000 μg) with 1 mM dATP, and 1-10 μM cytochrome *c* or purified caspases in a buffer containing 20 mM HEPES-NaOH (pH 7.0), 20 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$ and 10 mM DTT at 30˚C as previously described (27). Cell extracts were obtained as follows: The harvested cells were washed twice with ice cold PBS, resuspended in the lysis buffer shown in the SEC section on ice and dounce-homogenized with 40-50 strokes. Cell extracts were obtained from supernatants following microfuge centrifugation of the disrupted cells at 13,000 rpm for 1 h at 4˚C.

Construction and purification of caspases and Bid. Caspases-3 and -6, prodomainless equivalent of the p41/43 fragment of caspase-8 (p18-10) (see Results), and Bid were prepared as previously described (27). Prodomainless caspase-8 double mutant (D374A/D384A) mimicking the p30 fragment of caspase-8 (DM) (see Results) was constructed by point mutations using site-directed mutagenesis by polymerase chain reaction and DpnI-mediated cleavage methods (27,30). In the polymerase chain reaction, the template was caspase-8 cloned in the pET15b bacterial expression vector (Novagen, Darmstadt, Germany) and the primers were 5'-ATACC TGTTG AGACT GCTTC AGAGGA GCAA-3' (sense) and 5'-TTGCT CCTCT GAAGC AGTCT CAACA GGTAT-3' (antisense) for D374A; 5'-TATTT AGAAA TGGCT TTATC ATCAC CTCAA-3' (sense) and 5'-TTGAG GTGAT GATAA AGCCA TTTCT AAATA-3' (antisense) for D384A. The underlined sequences refer to the incorporated alanine sequence in each case. The mutated plasmid was confirmed by sequencing both strands. The recombinant caspase was expressed in a BL21 pLys *Escherichia coli* strain and purified as previously described (27). Aliquots of the purified proteins were stored at -80˚C until use. The amount of protein was measured through Bradford assay according to the instructions provided by the manufacturer (Sigma).

Results

Single treatment with Aβ42 induces the minimal activation of caspases-3, -8 and -9. We initially sought an experimental condition under which caspase would be robustly activated in Aβ-treated cells to probe the Aβ-induced apoptotic pathway. To achieve this, we sought to use Aβ-treatment methods that result in the activation. In this study, we examined the activities of caspases-3, -8 and -9, which participate in the IAPW and EAPW. Their activities were measured using three synthetic substrates: Ac-DEVD-AMC, Ac-IETD-AMC and Ac-LEHD-AMC for caspase-3, -8 and -9, respectively. The cells were incubated with up to 40 μM Aβ42 for up to 48 h (Fig. 1). The oligomeric preparation of Aβ42 was mainly used in the present study, as it is superior to the monomer or fibrillar form in inducing caspase activity and cell death (4,8). Unless otherwise indicated, Aβ42 refers to the oligomeric preparation of the peptide in the following experiments. In this experiment, we employed human epithelial HeLa cells that exhibited a relatively higher caspase activity than other cell lines, such as human neuroblastoma SH-SY5Y cells (26) in which death occurred too readily upon Aβ treatment and the levels of activated caspase were often varied (data not shown).

Some levels of caspase activity on the synthetic substrates were detected in the Aβ42-treated cells, particularly after 48 h of incubation (Fig. 1A-C). The activity levels were lower by several folds than those induced by other damaging agents, such as STS (Fig. 7A and B) (26). Although a number of studies, including ours have indicated the involvement of apoptosis and accompanying caspase activation in Aβ-induced cell death (9-12,26), low activities of caspases are observed, as shown in Fig. 1. Moreover, we were not convinced that even the low activity was caused by Aβ42-induced damage, as no Aβ42-concentration-dependent activity was observed.

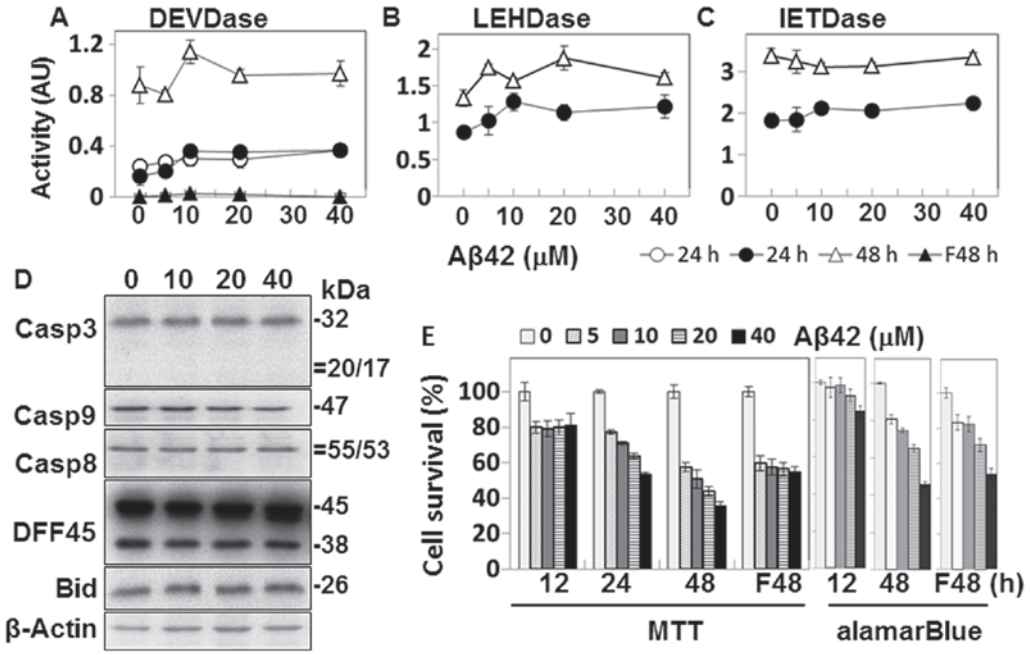


Figure 1. Activation of caspases, processing of their substrates, and cell death in amyloid-β (Aβ)42-singly-treated cells. (A-C) DEVDase, LEHDase, and IETDase activities were measured with 10 μM DEVD-AMC, 50 μM LEHD-AMC, and 50 μM IETD-AMC for cells treated with Aβ42 at the indicated concentrations and durations. AU, arbitrary unit. (D) Western blot analysis of caspases and their substrates was performed using cells treated with Aβ42 at the indicated concentrations for 48 h. β-actin was used as a loading control. The relative sizes are indicated on the right side of the figures. The result is representative of at least three independent experiments. (E) Cell death was estimated in cells treated with Aβ42 at the indicated concentrations and time-points using MTT reduction and alamarBlue assays. F48 indicates samples treated with fibrillary Aβ42 for 48 h. The results of (A-C and E) are expressed as the means ± standard deviation of 3 independent experiments. Casp, caspase.

The activation of caspases and the processing of DFF45 and Bid, substrates of caspases-3 and -8, respectively, were further explored in the Aβ42-treated cells by western blot analysis. Cleaved fragments or reduction in levels of the pro-proteins was expected if they are activated (Fig. 2). No such fragment or reduction was detected by western blot analysis (Fig. 1D). Taken together, our data show no evidence of caspase activation in the cells treated with Aβ42 under the experimental conditions described above.

Aβ42 cytotoxicity was examined to confirm that the low level of caspase activation was not due to faulty peptide preparation. Cell death was mainly assessed by MTT assay (27). However, soluble Aβ can lead to a decrease in MTT formazan production in the absence of overt cell death when cells are incubated for longer durations (35). Thus, the alamarBlue assay was also performed to complement the cell death experiments. Slight reductions in MTT formazan production were observed in samples after 12 h incubation (Fig. 1E). However, it is questionable whether cell death occurred in the samples, because they showed no Aβ42-dose dependency and the levels of cell death in these samples were barely decreased in the alamarBlue assay. On the contrary, in samples of 24- and 48-h incubation, MTT formazan production was reduced in an Aβ42-dose-dependent manner (Fig. 1E). Consistent with this, reduction in alamarBlue was observed in the 48-h sample, although the levels were less than those of the MTT assay (Fig. 1E). Based on the results of the 24- and 48-h samples, we concluded that the Aβ42 peptide used herein was cytotoxic.

It has been reported that Aβ fibrils can induce the activation of the EAPW, which leads to activation of caspase-8 and subsequently, caspase-3 (12). In the present study, caspase-3-like DEVDase activity was not detected in the fibrillar Aβ42-treated cells (Fig. 1A). MTT formazan production was reduced in the cells treated with the fibrillary form of Aβ42, but it was not Aβ42-dose-dependent (Fig. 1E). It seems that the ambiguous results were caused by the non-specific reduction of MTT formazan production. On the contrary, the Aβ-dose-dependent reduction was clearly observed in the alamarBlue assay on the same samples, suggesting that Aβ42-dose-dependent cell death occurred (Fig. 1E). Taken together, these data suggest that the fibrillar form of Aβ42 led to cell death in which caspase-dependent apoptosis played a minimal role in the process. In the following experiments, fibrillar Aβ42 was not used, due to the lack of caspase activation in the peptide-treated cells (Fig. 1A).

Double treatment with Aβ induces potent caspase activation. As Aβ42-dependent caspase activation was not prominent under the above-mentioned treatment conditions, we sought other conditions for the robust activation of the enzyme. We previously observed that the 'double' treatment of the peptide could lead to higher levels of caspase activation (27). Others have reported that nucleation-dependent polymerization in the doubly treated cells was essential for Aβ-mediated cell death (31,36). Thus, we hypothesized that the polymerization process in the doubly treated samples may provide a different or stronger signal to activate caspases. In the present study, we systematically tested the double treatment method using different combinations of conditions to find an optimal condition under which caspases could be potently activated. After testing different time points, a 2-h initial treatment of Aβ42 at the indicated concentrations was selected, as it resulted in the highest caspase activity (other time point data not shown). At

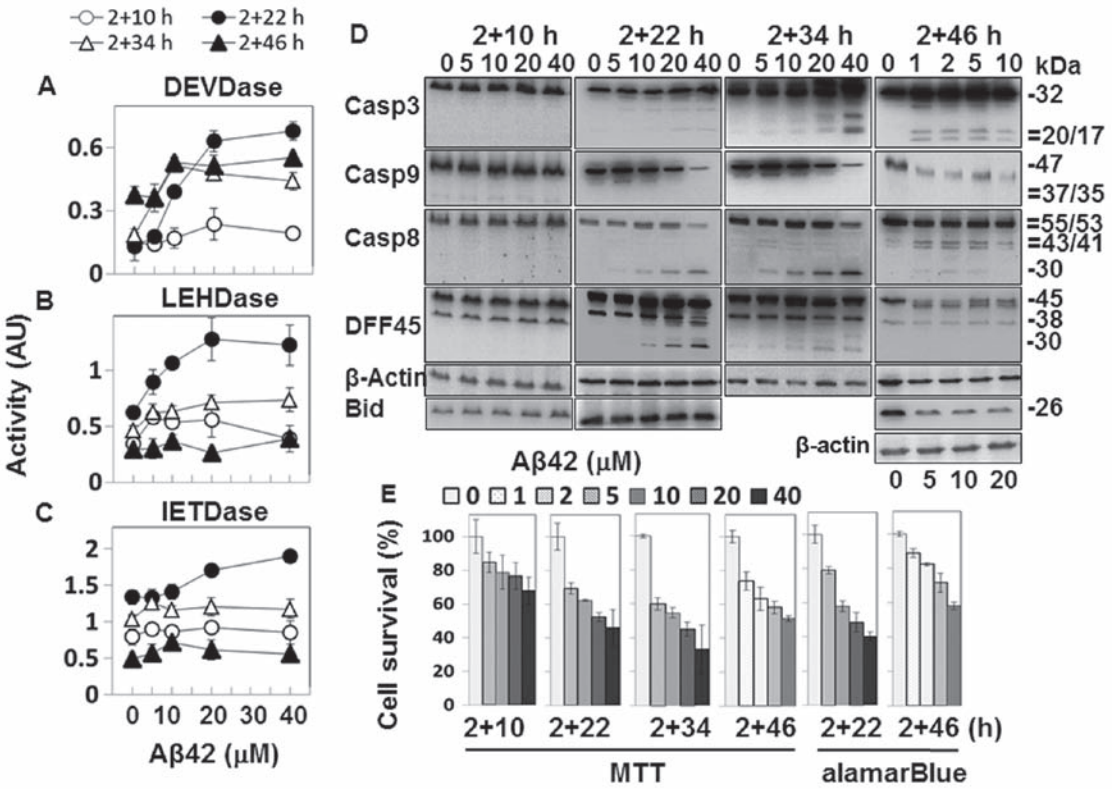


Figure 2. Activation of caspases, processing of their substrates, and cell death in amyloid-β (Aβ)42-doubly-treated-cells. (A-C) Enzyme activities were determined as described for Fig. 1A for cells doubly treated with Aβ42 at the indicated concentrations and time. (D) Western blot analysis of caspases and their substrates was performed using cells treated with Aβ42 at the indicated concentrations and time-points. β-actin was used as a loading control. The relative sizes are indicated on the right side of the figures. The result is representative of at least three independent experiments. We used lower concentrations of Aβ42 (up to 20 μM for Bid and 10 μM for others) in the 2+46 h groups, because the higher concentrations of the peptide resulted in severely damaged cells with which the immunoblot analysis was difficult to perform (data not shown). (E) Cell death was estimated in cells treated with Aβ42 at the indicated concentrations and time-points using MTT reduction and alamarBlue assays. The results of (A-C and E) are expressed as the means ± standard deviation of 3 independent experiments.

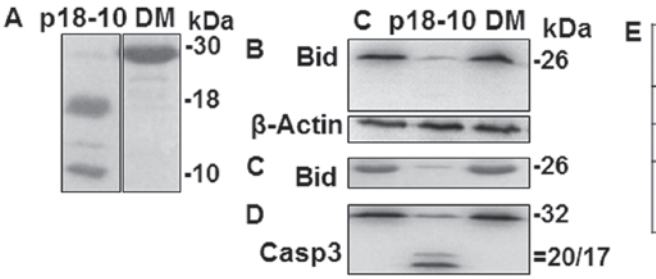
the end of the first incubation, culture medium containing the peptide was removed, and subsequently, the cells were further incubated with a new preparation of Aβ42 peptide at the same concentration for 10, 22, 34 or 46 h (2+10 h, 2+22 h, 2+34 h, or 2+46 h sample, respectively). It is noted that in a previous study (36), cells were treated with the fibrillar form of Aβ42 for 1 h followed by treatment with soluble Aβ42; however, in this study, we used only soluble oligomeric Aβ42, as this resulted in robust caspase activation (Fig. 2A-C).

The samples with 2+22 h and longer incubation durations showed caspase-3-like DEVDase activity in an Aβ42-dose-dependent manner (Fig. 2A). A processed fragment of ~20 kDa was detected in the 2+22 h and 2+34 h samples treated with high concentrations of Aβ42, and the fully processed ~17 kDa fragment was recognized in the 2+46 h samples in addition (Fig. 2D). Although the band intensity of the processed fragment was the strongest at 2+34 h, and the additional ~17-kDa fragment was present in the 2+46-h samples (Fig. 2D), DEVDase activities of these samples were similar to that of the 2+22 h sample (Fig. 2A), indicating that the activity and the level of observed fragment processing were not closely correlated. A reduction in DFF45, a substrate of caspase-3, was also observed (Fig. 2D), confirming the catalytic activation of caspase-3.

The Aβ42-dose-dependent caspase-9-like LEHDase was prominently activated in the 2+22 h sample, and to a lesser degree, in the samples with longer incubation durations (Fig. 2B). The levels of procaspase-9 were decreased in the 2+22 h sample and in samples with longer incubation durations treated with high doses of Aβ42 (Fig. 2D). These data support the activation of caspase-9. The processing of procaspase-3 (Fig. 2D), the substrate of caspase-9, further supports catalytic activation of caspase-9. Cleaved products of ~35/37 kDa, detected in the analysis of apoptosome (Fig. 4C), were not detected in the samples (Fig. 2D), possibly due to insufficient intensity of the immunoblot signals.

Caspase-8-like IETDase activity was also the highest in the 2+22 h sample, although the correlation between Aβ42-dose and the activity was weak (Fig. 2C). Procaspase-8 was processed in the 2+22 h sample and samples with longer incubation durations, but two different types of fragments were detected depending on the incubation duration (Fig. 2D). In the 2+22 h samples, a fragment of ~30 kDa (p30) was largely detected, while the typical 41/43 kDa fragments (p41/43) were detected in the 2+46 h samples. p30 is a fragment of procaspase-8 lacking the prodomain (37), while the p41/43 fragment contains the prodomain and the large domain which becomes the large subunit when caspase-8 is fully maturated. Thus, the cleavage sites to form the two different fragments are distinct (37).

In order to determine the cytotoxicity of the Aβ42 peptide preparation, cell death was assessed in the double treatment samples. All the samples tested showed Aβ42-dose-dependent cell death (Fig. 2E). The levels of cell death evaluated by MTT-formazan reduction were generally higher than those by



E

Enzyme	p18-10	DM
K_M (mM)	29.86	28.78
k_{cat} s^{-1}	0.08	0.000098
k_{cat}/K_M ($M^{-1}S^{-1}$)	2680	3.4

Figure 3. Activities of purified caspase-8 variants. (A) Purified p18-10 and DM are shown using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). (B and C) Processing was examined for Bid from cell extracts (70 μg) (B) or purified Bid (1 μg) (C) that was incubated with 30 ng of p18-10 or DM for 1 h. The proteins were visualized by western blot analysis (B) or SDS-PAGE (C). The letter 'C' indicates a control sample without p18-10 or DM. β-actin was used as a loading control. (D) Procaspase-3 processing was also probed as described in (B). (E) Catalytic parameters of purified recombinant p18-10 and DM were calculated using the Michaelis-Menten equation (34) after quantification of the activities using IETD-AMC as a substrate. Casp, caspase.

alamarBlue assay (2+22 h and 2+46 h samples of MTT vs. those of alamarBlue), as seen in the single treatment samples (Fig. 1E). Reductions in the values were slightly higher in the double treatment samples than in the equivalent single treatment samples (i.e. 2+22 vs. 24 h). The single and double treatment samples generally exhibited similar levels of cell death.

Catalytic activity of p30 and p41/43 on Bid processing. Bid, a substrate of active caspase-8 (38), was not cleaved in cells transfected with a plasmid encoding a p30-equivalent enzyme (39) and the catalytic activity of the purified p30-equivalent enzyme was weak (39-41). In the 2+22 h samples which contained p30, the immunoblot signal intensity of Bid was similar to that of control samples without Aβ42 treatment (Fig. 2D). Thus, it is reasonable to assume that p30 cannot cleave Bid. To confirm this, we determined whether p30 would process Bid, using a purified prodomainless caspase-8 double mutant protein mimicking p30 (DM) (see Materials and methods) (Fig. 3A) (42). The catalytic activity was compared with that of the enzyme containing the prodomainless equivalent of p41/43 (p18-10) (Fig. 3A), which is known as the major enzyme for Bid processing (43). Purified p18-10 was able to process procaspase-3 and Bid from crude cell extracts and purified Bid as expected, while DM exhibited inefficient processing (Fig. 3B-D). The k_{cat} of DM on IETD-based synthetic substrate was ~800-fold lower than that of p18-10 (Fig. 3E), indicating that the lower activity of DM was due to the slower catalytic velocity, consistent with the previous studies (39,40,42).

Bid reduction was observed in the cells incubated for 2+46 h with Aβ42 at concentrations higher than 5 μM (Fig. 2D). The timing of p41/43 generation correlated well with the reduction in Bid, indicating that catalytic activation of caspase-8 was responsible for Bid processing, as reported previously (38). It was noted that production of p41/43 and Bid reduction were observed after activity of the caspase-3 (substrate of caspases-8 and -9)-like DEVDase reached its highest level (2+46 h samples) (Fig. 2A).

Cytochrome c release from the mitochondria and the formation of the Apaf-1 apoptosome in the Aβ42-doubly-treated cells. Catalytic activity and the processing of caspases-9 and -3 in the Aβ42-doubly-treated cells (Fig. 2A-D) suggest that the IAPW was activated in the cells. To confirm the activation of the pathway, cytochrome *c* release from the mitochondria was probed in the 2+22 h samples that showed potent activation and processing of caspase-3, and reduction of procaspase-9 (Fig. 2A-D). cytochrome *c* was detected in the soluble fraction of cell extracts prepared from the samples treated with 20 and 40 μM Aβ42, evidencing the release of the protein in corresponding cells (Fig. 4A). Subsequently, the formation of apoptosome, a hallmark of the IAPW, was examined in the 2+22 h sample treated with 20 μM Aβ42. Apaf-1 and caspase-9 were detected only in the later fractions (>25) of SEC in the control sample prepared from Aβ42-non-treated cells (Fig. 4B) or cells treated once with Aβ42 for 24 h (Fig. 4C), whereas they were detected in the earlier fractions (~15-23) in the sample treated with 20 μM of Aβ42 for 2+22 h (Fig. 4C). These data clearly indicated the formation of apoptosome in the 2+22 h sample. Thus, we concluded that IAPW was activated in the cells. It was noted that the 35/37 kDa fragments of caspase-9, which were not detected in the total lysate (Fig. 2D), were clearly detected in the western blot analysis following SEC (Fig. 4C). They were detected in this analysis, probably since the protein was concentrated during chromatography.

The Aβ42-induced activation of the IAPW was compared with the previous results obtained from the cells treated with STS, which has been employed as an IAPW-inducing agent (27). The levels of cytochrome *c* recovered in the supernatant fraction were low (~40% of total cytochrome *c* protein) in the 2+22 h sample treated with 20 or 40 μM of Aβ42 (Fig. 4A), compared with that of STS-treated cells (~90%) (Fig. 5A). Cytochrome *c* is an essential constituent of apoptosome. Thus, the reduced level of released cytochrome *c* by Aβ42 compared to STS could negatively influence robust activation of IAPW (see Discussion).

The apoptosome formed in the sample treated for 2+22 h with 20 μM Aβ42 (Fig. 4D) also differed from that of the STS-treated cells in terms of unprocessed and processed caspase-9 abundance in the apoptosome complex. Unprocessed procaspase-9 (47 kDa) comprised the majority (~34% of total caspase-9 protein) of caspase-9 (~40%) bound to the protein complex formed in the 2+22 h Aβ42 treated sample (Fig. 5B), whereas only processed caspase-9 (35/37 kDa) was detected in the complex of STS-treated cells (17% of total caspase-9 protein) (Fig. 5B). Consistently, the level of processed caspase-9 was much lower in the 2+22 h samples than in STS-treated cells (11 vs. 65%) (Fig. 5B). Cell-free experiments were performed as an attempt to understand the differential caspase-9 processing

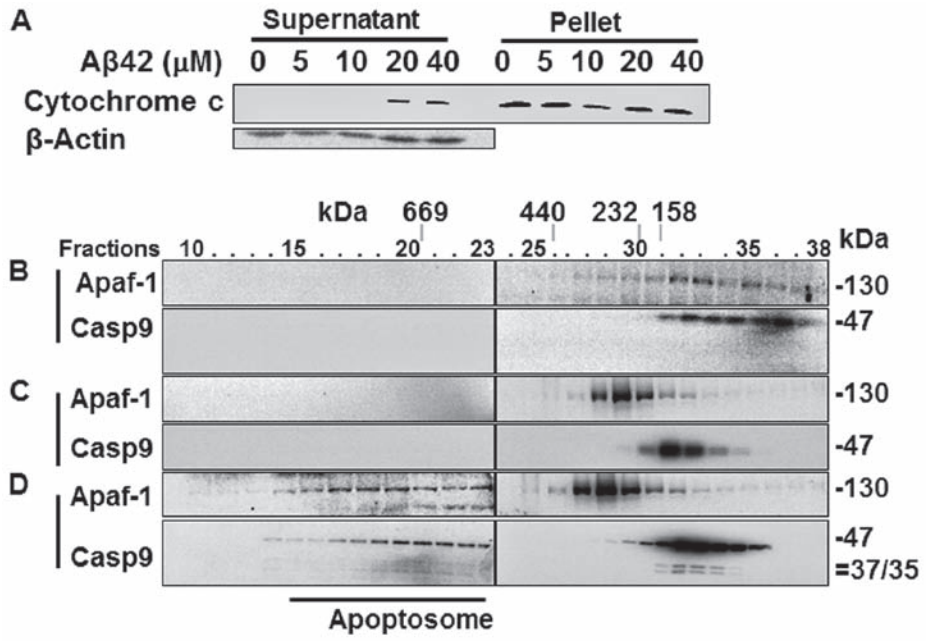


Figure 4. Cytochrome *c* release and the formation of the apoptotic protease activating factor-1 (Apaf-1) apoptosome in amyloid-β (Aβ)42-doubly treated cells. (A) Cytochrome *c* release was assessed by western blot analysis of soluble (supernatant) and precipitated (pellet) parts of the extracts prepared from cells treated with 20 μM Aβ42 for 2+22 h. β-actin was used as a loading control. (B-D) The extracts (1 mg) prepared from the cells incubated without (B) or with 20 μM Aβ42 for 24 h (C) or 2+22 h (D) were fractionated by size exclusion column chromatography (SEC). The fractions were then analyzed by western blot analysis for Apaf-1 and caspase-9. The standard molecular markers and elution fractions are indicated above the images. The relative sizes are indicated on the right side of the figures. The results are representative of at least 3 independent experiments. Casp, caspase.

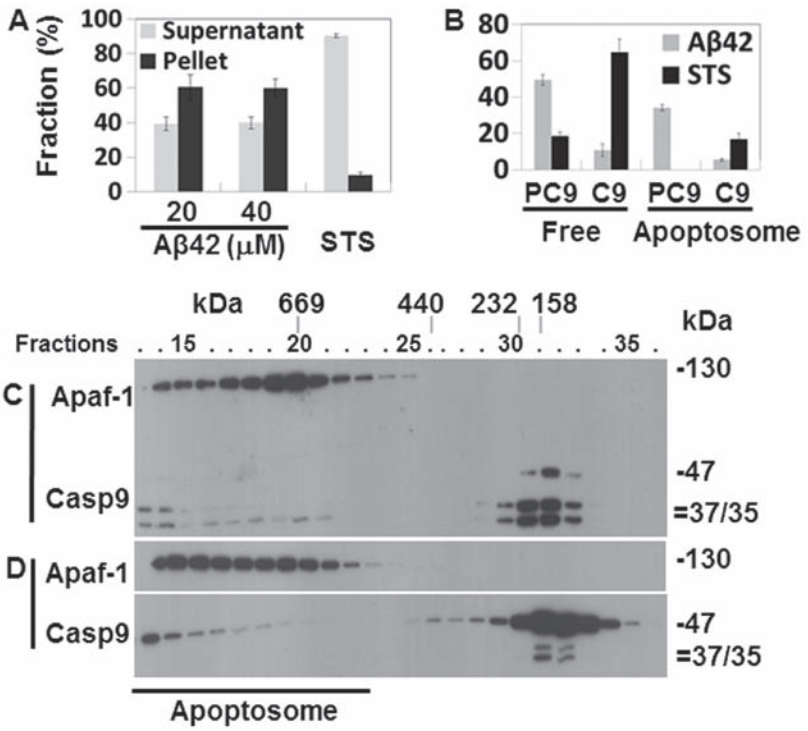


Figure 5. Comparison of released cytochrome *c* and processing of procaspase-9 in staurosporine (STS) and the 2+22 h amyloid-β (Aβ)42-treated samples and cell-free formation of apoptosome. (A and B) Optical densities of immunoreactive bands of cytochrome *c* and caspase-9 from Fig. 4A and D were quantified and compared with those obtained in STS-treated cells which were adopted from ref. (27). The results are expressed as the means ± standard deviation of 3 independent experiments. PC9 and C9 indicate procaspase-9 and processed caspase-9, respectively. (C and D) Cell extracts (1 mg) prepared from healthy cells were treated with 1 mM dATP and 1 μM cytochrome *c* in the absence (C) or presence of 20 μM Aβ42 (D) for 1 h and fractionated by size exclusion column chromatography (SEC). And then, the fractions were analyzed as described for Fig. 4B-D. The standard molecular markers, elution fractions and the releative sizes were indicated as shown in Fig. 4B-D. The results are representative of at least 3 independent experiments. Casp, caspase.

in the two samples (STS-treated cells vs. 2+22 h sample treated with Aβ42). Apoptosome formed in the cell lysate treated with dATP and cytochrome *c* contained only processed 35/37 kDa caspase-9 as that formed in the STS-treated cells (Fig. 5C),

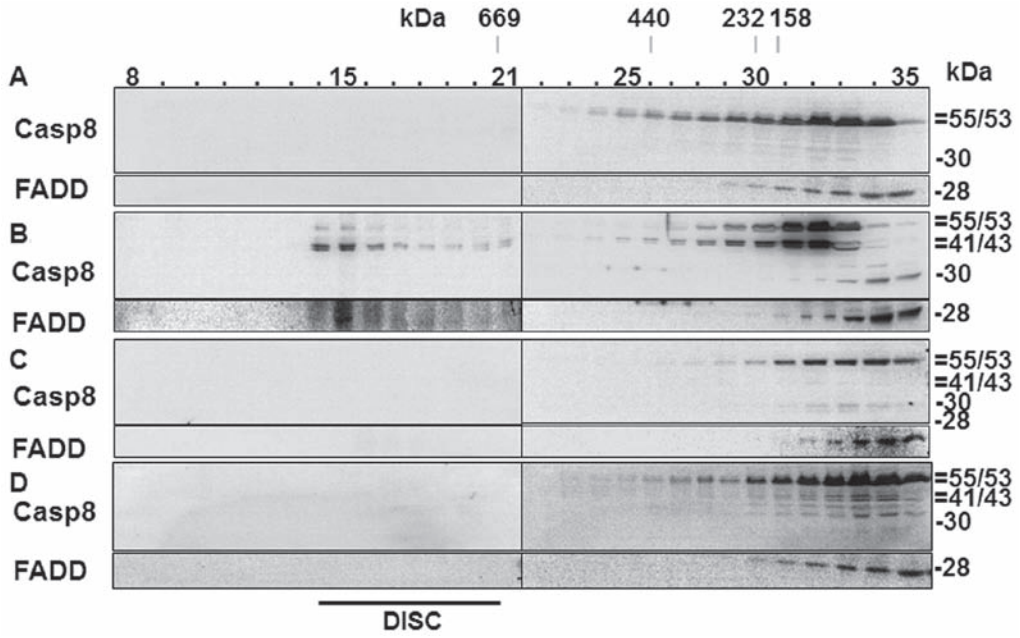


Figure 6. Absence of death-inducing signaling complex (DISC) assembly in amyloid-β (Aβ)42-doubly-treated cells. Cells were incubated without (A), with 100 μg/ml of actinomycin D for 2 h and 50 nM of tumor necrosis factor-α (TNF-α) for another 24 h (B), with 40 μM Aβ42 for 2+22 h (C), or 10 μM Aβ42 for 2+46 h (D), respectively. Cell extracts prepared from the cells (1 mg) were fractionated by size exclusion column chromatography (SEC) and the fractions were analyzed by western blot analysis for caspase-8 and FADD. The standard molecular markers and elution fractions are indicated above the figures. The relative sizes are also indicated on the right side of the figures. The results are representative of at least 3 independent experiments. Casp, caspase.

whereas the apoptosome formed by dATP and cytochrome *c* in the presence of 20 μM Aβ42 contained only unprocessed procaspase-9 (Fig. 5D). These data clearly indicate that Aβ42 inhibited procaspase-9 processing in the apoptosome. It is noted that more caspase-9, processed or unprocessed, was detected in apoptosome of the 2+22 h sample (~40%) than in STS-treated cells (~17%), implying that suppressive effect of Aβ42 on the formation of apoptosome by binding to procaspase-9 (27) was overcome. Taken together, it seems that IAPW may not be fully activated by Aβ42 as substantiated by inefficient catalytic processing of procaspase-9 (and reduced levels of released cytochrome *c*) which could be the cause of the low response (such as caspase activation) of cells to the peptide (Fig. 2).

DISC is not formed in Aβ42-treated cells. A number of studies have indicated that EAPW plays a crucial role in the apoptotic process induced by Aβ42 (13,14,44). In the present study, we observed activation of caspase-8 and Bid processing (Fig. 2D), which are hallmarks of EAPW activation in the type II cells such as HeLa cells used in the present study (45). Another hallmark of the pathway is the formation of DISC (34,37), which has not been shown in Aβ42-treated cells previously. We probed formation of DISC in the Aβ42-doubly-treated cells in the present study to confirm its involvement in the cells. In the SEC analysis of control cells which were not treated with any cell death agent, procaspase-8 and FADD proteins were detected only in the later fractions (>22) (Fig. 6A), while fractions ~14-21 contained the two proteins in a positive control sample prepared from cells treated with actinomycin-D followed by tumor necrosis factor-α (TNF-α) (Fig. 6B). The two proteins recovered in the early fractions (~14-21) are components of DISC (13).

In the cells incubated for 2+22 h with 40 μM Aβ42, which showed the highest levels of DEVDase, LEHDase and IETDase activities, and the production of processed caspases (Fig. 2A-D), procaspase-8 and FADD were detected only in the later fractions (>22) (Fig. 6C), indicating that DISC was not formed in those samples. DISC formation was further examined in the cells incubated for 2+46 h with 10 μM Aβ42, in which the generation of the p41/43 caspase-8 fragment, Bid reduction, and IETDase activation were evident (Fig. 2A-D). DISC was also not detected in these cells (Fig. 6D). Thus, we hypothesized that the formation of p41/43 and Bid processing in the cells incubated for 2+46 h with 10 μM Aβ42 were through a pathway other than EAPW. Higher concentrations (>20 μM) of Aβ42 could not be employed in the experiment with 2+46 h incubation to probe the presence of DISC by SEC, as the cells were so severely damaged that it was difficult to obtain a sufficient amount of protein from these cells (data not shown). It was noted that p30 was present in the 2+22 h sample (Fig. 6C), consistent with that observed in Fig. 2D, while p41/43 was additionally detected in the 2+46 h sample (Fig. 6D). These data confirm that the formation of the p30 fragment of caspase-8 precedes that of the p41/43 fragment.

Fragmentation of procaspase-8 into p30 and p41/43 in the cell-free apoptotic process. Since DISC was not formed in the cells doubly treated with Aβ42 (Fig. 6C and D) and that caspase-3 activation preceded caspase-8 activation and Bid cleavage (Fig. 2A-D), we speculated that caspases-3 and -6 activated in the IAPW (46-48) is responsible for the formation of p30 and p41/43. Caspase-3 was activated in the 2+22 h sample with 20 μM Aβ42 as shown in Figs. 2A and D, and 6A and B. Caspase-6 activation in the cells was verified by catalytic activation with a VEID-based synthetic substrate and the production of a ~23-kDa caspase-6 fragment (Fig. 7A and B).

To confirm and characterize the fragmentation of procaspase-8 in the IAPW, we employed a cell-free experimental

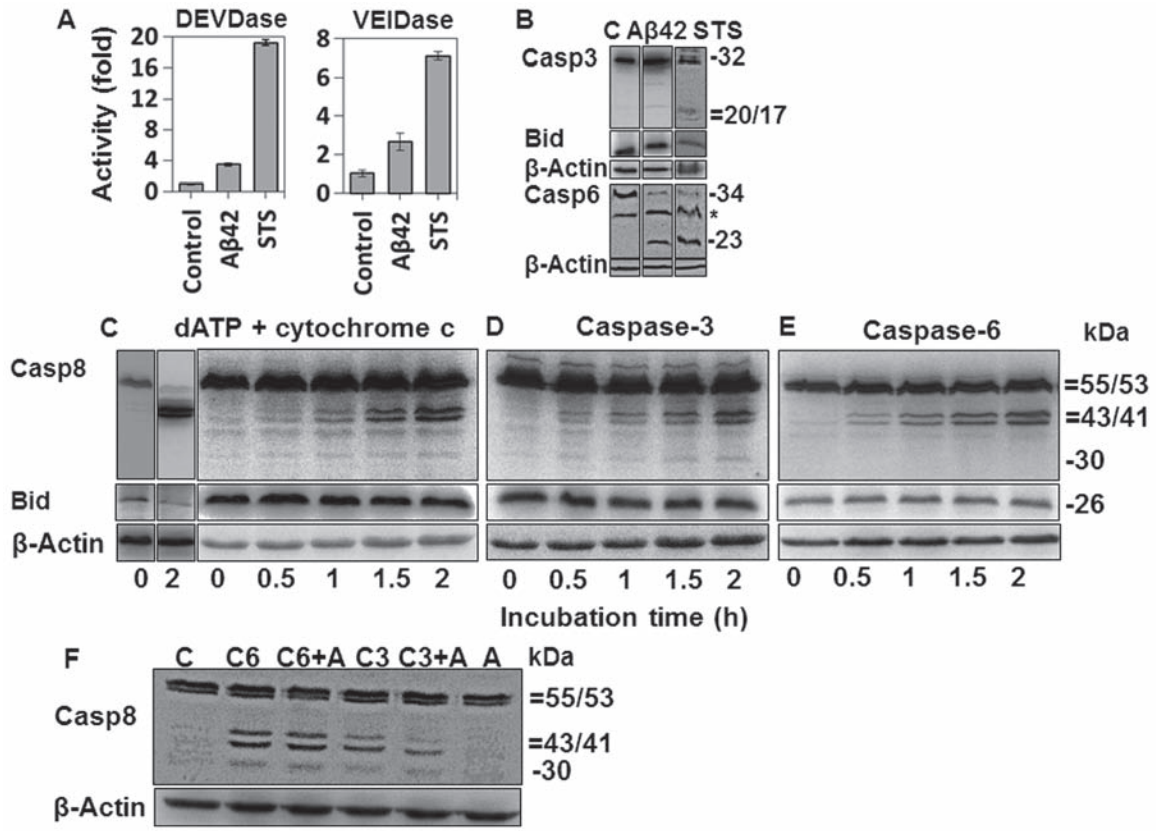


Figure 7. Activation of caspases-3 and -6 in amyloid-β (Aβ)42-doubly-treated or staurosporine (STS)-treated cells and fragmentation of procaspase-8 in cell-free apoptotic process. (A) Activities of caspases-3 and -6 were measured for cells treated with 20 μM Aβ42 for 2+22 h or with 0.1 mM STS for 6 h, using 10 μM DEVD-AMC and 50 μM VEID-AMC as substrates. Activity is indicated by fold increase over that of the control and expressed as mean ± standard deviation of 3 independent experiments. DEVDase data of the Aβ42 group was extracted from Fig. 2A. (B) Cleavages of procaspase-3, bid, and procaspase-6 were probed by western blot analysis for the cells treated as described in (A). *, Denotes a nonspecific band. C, indicates untreated control. (C-E) Cell extracts were incubated with cytochrome *c* (10 μM for lane 2 and 1 μM for 3-7 lanes) and 1 mM dATP (C), purified caspase-3 (30 ng) (D) or purified caspase-6 (50 ng) (E) for the indicated durations and analyzed by western blot analysis for caspase-8 and bid. (F) Cell extracts (70 μg) were incubated with purified caspase-6 (50 ng) or caspase-3 (30 ng) in the presence or absence of 20 μM Aβ42, and analyzed for procaspase-8 cleavage as in Fig. 6C-E. (B-F) β-actin was used as a loading control. The relative sizes are indicated at the right side of the figures. The results are a representative of at least 3 independent experiments. Lane 1 in (B-E) and in lane C of (F) are a control sample prepared from cell extracts incubated without cytochrome *c*/dATP or caspases. C3, C6 and A, indicate caspase-3, caspase-6 and Aβ42, respectively. Casp, caspase.

approach, in which cell extracts were incubated with 1 mM dATP and 10 μM cytochrome *c*, leading to the formation of the Apaf-1 apoptosome (27) or with purified caspases-3 and -6. As expected, procaspase-8 and its substrate Bid were processed in the samples containing dATP and cytochrome *c*, but only p41/43 was detected (Fig. 7C, second lane). The p30 fragment was seen in the following experiments in which a lower dose (1 μM) of cytochrome *c* was used (Fig. 7C, lanes 3-7). In these samples, procaspase-8 processing was incomplete, suggesting that p30 might exist only in the early stage of apoptosis, consistent with the results of Figs. 2D and 5C and D.

We added purified caspases-3 and -6 to the prepared cell extract, and examined whether each fragment of caspase-8 was generated, to determine which enzyme was responsible for the formation of each caspase-8 fragment. Each purified enzyme was added at a concentration lower than that which can cause complete fragmentation of procaspase-8, to reproduce the results of the cell-based experiments (Fig. 2D). Purified caspase-3 could induce the formation of both p30 and p41/43 fragments (Fig. 7D), while caspase-6 induced only the formation of p41/43 fragment (Fig. 7E). The cell-free samples shown in Fig. 7C-E seemed to reflect the situation of the samples incubated for 2+22 h with 20-40 μM Aβ42, as shown in Fig. 2D, in consideration of the level of Bid and processed caspase-8. Taken together, data from the cell-free experiments confirm that both p30 and p41/43 fragments could be formed through the IAPW, although physiological implication of the differential activities of caspases-3 and -6 on procaspase-8 processing remains to be investigated.

In the present study, we demonstrated that the IAPW was activated in Aβ42-doubly-treated cells. However, it was noted that DEVDase and VEIDase activity, and processing of the two caspases were less in the Aβ42-treated cells than those in STS-treated cells by several fold (Fig. 7A and B). The limited activation of caspases-3 and -6 in the Aβ42-treated cells seemed to be responsible for the incomplete activation of caspase-8 (Figs. 2D and 6C-E). On the other hand, it is also possible that Aβ42 could negatively affect the activity of caspases-3 and -6 for the processing of procaspase-8. To test this hypothesis, the effect of Aβ42 on the formation of p30 and p41/43 was explored in the cell-free samples with purified caspases-3 and -6 proteins. Caspase-6 generated similar levels of p30 and p41/43 fragments independent of Aβ42 treatment, while caspase-3 appeared to be only slightly affected by the

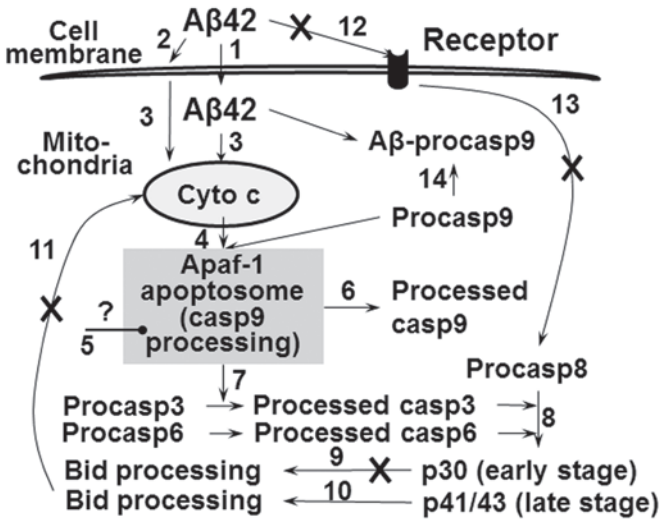


Figure 8. Summary of proposed model for the effect of Aβ on apoptosis pathway. Amyloid-β (Aβ)42 enters into cell (1), interacts with cell membrane or forms a channel-like structure on cell membrane (2) to induce intrinsic apoptosis pathway (IAPW) (3). Cytochrome *c* (cyto *c*) is released from the mitochondria in the pathway (4) and forms apoptosome with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9. Caspase-9 is processed in the protein complex (5) and a part of the processed enzyme is released from it (6). The Apaf-1 apoptosome processes procaspase-3 to caspase-3 which can activate caspase-6 (7). Caspases-3 and -6 cleave procaspase-8 to produce p30 and p41/43 (8). p30 cannot cleave Bid (9) and p41/43 is generated in the late stage of apoptosis (10). Thus, the amplification loop may not be fully functional (11). Death-inducing signaling complex (DISC) is not formed in the Aβ42-treated cells (12) and caspase-8 appears to be activated by IAPW (8) instead of extrinsic apoptosis pathway (EAPW) (13). Aβ42 can interact with procaspase-9 to suppress the apoptosome formation (14). However, in 2+22 h sample with 20 μM Aβ42 the suppression seems to be overcome as evidenced by the formation of the protein complex in the cells (Fig. 4D). It is suggested that the response to Aβ treatment is lower than that of staurosporine (STS) treatment, because of lower level of cytochrome *c* release (Fig. 4, step 4), less processing of procaspase-9 (Fig. 4, step 5) and absence of the amplification loop (Fig. 7, step 11) in Aβ42-treated cells. Casp, caspase.

peptide in producing the fragments (Fig. 7F). Thus, it was concluded that the lower levels of caspases-3 and -6 activation, but not their activity, were responsible for the incomplete activation of caspase-8 in the Aβ42-doubly-treated cells (Fig. 2A-C). This is consistent with our previous study demonstrating that Aβ42 did not affect the activities of caspases except on procaspase-9 (27).

Discussion

Although caspase is not robustly activated in Aβ-treated cells (Figs. 1 and 6A and B) (26), and Aβ42 can suppress IAPW by interacting with procaspase-9 (27), accumulating data, including ours, have implicated the apoptotic process as a mechanistic feature of the cell death induced by Aβ42 (19,49). In the present study, we sought a caspase-activating condition in Aβ42-treated cells and explored the apoptosis pathways involved under the condition to assess cytotoxic properties of Aβ42 and its mechanistic features. To achieve this, we explored various culture systems and methods associated with Aβ42 treatment in the experiments.

Caspase activity was detected in the cells treated 'once' with Aβ42 in the previous studies (9,12). In the present study, the activity was also detected in the cells singly treated with the peptide (Fig. 1A-C). However, Aβ42-dose dependency of the activity was not observed (Fig. 1A-C) and furthermore, the processed fragments of caspases were not detected (Fig. 1D). In fact, detection of processed fragments of caspases may not be necessary to prove the activation of the enzyme, because an undetectable amount of activated caspase can exhibit catalytic activity with the synthetic substrates (data not shown). Thus, we think that a certain low level of caspase activation could have occurred in the single treatment samples. However, it is questionable whether the activity was elicited solely by the addition of Aβ42. For example, it is possible that serum deprivation of the cells before Aβ42 treatment caused increased background activity. Thus, it is not clear whether the activity was due to Aβ42 treatment or other unknown factors.

The activation of caspase was observed in cells 'doubly' treated with Aβ42 in which Aβ42-dose dependency of the activity was evident, and the processed fragments of caspases or reduction of procaspase was detected (Fig. 2A-D). The Apaf-1 apoptosome was also formed in the Aβ42-doubly-treated cells (Fig. 4B), suggesting that the IAPW was activated in the cells. Polymerization of the peptide (50) likely provides a strong signal to activate the apoptotic pathway and related caspases, although the mechanism underlying the necessity of the double treatment of Aβ42 to induce the activation of caspases remains poorly understood. The suppressive effect of Aβ42 on the formation of the apoptosome (27) seemed to be overcome in the cells. Relatively longer exposure (2+22 h in the present study vs. 4+6 h) (27) of cells to Aβ42 might be an inducing factor for the formation of apoptosome.

However, Aβ42-induced IAPW was different from that of induced by other agent (STS) in that less cytochrome *c* was released from the mitochondria and the majority of procaspase-9 in the Apaf-1 complex was not processed in the peptide-treated cells (Figs. 4D and 5). Previously we showed that Aβ42 inhibited activity of purified procaspase-9 (27), consistent with the present observation. On the other hand, we doubt that the lower level of cytochrome *c* in the 2+22 h sample (Fig. 5A) negatively affected the processing of procaspase-9, because our previous data showed that cytochrome *c* concentration variation to a certain extent did not lead to differential processing of caspase-9 (27). It is speculated that processing efficiency of caspase-9 was high in the STS-treated cells and the lower affinity of processed caspase-9 for Aβ42 (27) led to the lower level of caspase-9 bound to apoptosome in the cells (Fig. 5B). We suggest that processing of procaspase-9 in the apoptosome is the key difference in Aβ42 and STS-induced IAPW than binding of the protein to the Apaf-1 apoptosome.

Some studies have indicated that EAPW is elicited in Aβ-treated cells (12,44). This conclusion is usually based on activation of caspase-8, cleavage of Bid, upregulation of related proteins, and downregulation of EAPW-inhibiting proteins in the cells. However, the formation of DISC, one of the essential processes of the EAPW, has not been shown in these studies, to the best of our knowledge. To confirm the involvement of EAPW in Aβ-induced apoptosis, DISC formation was explored in selected samples in the present study. However, we could not detect DISC formation in the 2+22 h sample with 40 μM Aβ42, in which caspase-3-like DEVDase and other caspase-related activities reached their highest levels (Figs. 2A-D and 5C). Furthermore, DISC formation was not seen in the 2+46 h

sample with 10 μM Aβ42, in which p41/43 production and Bid cleavage, main processes that DISC formation leads to, occurred (Figs. 2D and 5D). DISC may be formed in cells under other treatment conditions, for example, with longer exposure of cells to Aβ42, at higher concentrations. However, the role of the hypothetical DISC formation and EAPW activation in Aβ42-induced apoptosis are still questionable, because the apoptotic processes could be completed in the cells without DISC, as evidenced in the 2+22 h samples with 40 μM Aβ42 (Figs. 2A-D and 5C). It was expected that DISC would be formed before activation of caspase-3-related activity if EAPW played a crucial role in the induced apoptosis. Taken together, the formation of DISC and the activation of EAPW were not observed in cells in which Aβ-induced apoptosis occurred to a significant extent. Therefore, it is questionable whether they played active roles in the Aβ-induced apoptotic process.

It is reasonable to assume that the p41/43 fragments of caspase-8 was generated by IAPW in the cells, because DISC formation was absent in the selected samples (Fig. 6D). We confirmed that p41/43 was produced in the cell-free experiments in which IAPW was reconstituted by addition of dATP and cytochrome *c* to the cell extracts, consistently with previous studies (33,48) (Fig. 7C). In IAPW, p41/43 is produced by catalytically activating the cleavage of procaspase-8 by caspases-3 and -6 (46,51). The low levels of p41/43 in the Aβ42-doubly-treated cells, therefore, appear to be attributable to the lower activation of the caspases-3 and -6 (Fig. 7A and B), which could be a result of the inhibitory effect of Aβ42 on the formation of the Apaf-1 apoptosome, as reported previously (27). One of the results of the incomplete activation of caspase-8 is the lower level of Bid processing, as shown in Aβ42-doubly-treated cells (Fig. 2D). The level of Bid processing may not be sufficient to cause proper operation of the amplification loop, which acts as a positive feedback to reinforce the apoptotic death process in the type II cells such as HeLa cells used here (45). The reduced activity of caspases and lack of the amplification loop in the corresponding cells imply limited activation of IAPW.

The p30 fragment of caspase-8 was also detected in the Aβ42-treated cells (Fig. 2D). The presence of the caspase-8 fragment p30 has rarely been demonstrated in previous studies. It was generated in cells treated with CD95 by formation of DISC and further processed into p18 and p10 fragments through subsequent autocatalytic processes (37). Here, we showed for the first time that p30 was formed in the cells doubly treated with Aβ42 without formation of DISC (Fig. 6C and D). It could be produced in the cell-free assay with reconstituted IAPW (Fig. 7C-E), suggesting a potential role of the pathway in generating the fragment. p30 was catalytically inefficient in processing procaspase-3 and Bid (Fig. 3B-D). Thus, the physiological meaning of p30 formation in Aβ42-treated cells presently remains unknown. It is hypothesized that p30 can sensitize cells toward apoptosis as suggested previously (37), based on the observation that it was produced at earlier times of the treatment prior to formation of the p41/43 fragments (Fig. 2D). Proposed model for the effect of Aβ42 on apoptosis pathway was summarized in Fig. 8.

A previous study showed that the double treatment of Aβ42 was necessary to elicit the cell death (31). Many studies, however, have indicated that the single treatment was able to induce cell death. In the present study, cell death evaluated by MTT and alamarBlue assay occurred in a dose-dependent manner in the cells singly treated with Aβ42 (Fig. 1E). Similar levels of cell death were observed in cells doubly treated with the peptide (Fig. 2E). Remarkably, the apoptotic process accompanying caspase activation was observed only in the Aβ42-doubly-treated cells (Fig. 2), but not in the singly treated cells. These results imply that the cell death could occur via distinct pathways in the two different samples. Study to elucidate the differential death pathway for each case is ongoing. It is difficult to imagine that neuronal cells are exposed to Aβ42 just once in the affected patient. Thus, the experimental condition with two or more treatments of the peptide to cells may reflect more accurately the actual physiological conditions, and accordingly, it can be suggested that IAPW plays a role in the Aβ-induced cell death despite its limited activation. It is to be hoped that comprehensive characterization of the nature of the multiple treatments and related cell death pathway will provide novel insight into Aβ-associated pathology and control of AD.

Acknowledgments

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