

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

Md. IMAMUL ISLAM¹, Md. GOLAM SHAROAR^{1,4}, EUN-KYOUNG RYU² and IL-SEON PARK^{1,3}

¹Department of Medical Sciences, Chosun University, Gwangju 501-759; ²Department of Nursing, Kongju National University, Kongju 314-701; ³Department of Cellular and Molecular Medicine, Chosun University, Gwangju 501-759, Republic of Korea

Received July 29, 2016; Accepted October 3, 2017

DOI: 10.3892/ijmm.2017.3193

Abstract. Amyloid- β (A β), 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 A β treatment. In this study, we investigated whether these suppressive effects of A β can be overcome; we also examined the death-related pathways. Single treatments of cells with A β 42 for up to 48 h barely induced caspase activation. In cells treated with A β 42 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 A β 42-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 A β 42. 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 A β -treated cells. Taken together, our data suggest that the IAPW, albeit functional only to a limited extent, plays a major role in A β 42-induced apoptosis without the EAPW.

Introduction

Amyloid- β (A β) 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 A β 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 A β peptides are progressively deposited in the brain parenchyma and cerebral blood vessels (7). However, the soluble A β 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 A β 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 apoptotic pathway (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 multiprotein 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

Correspondence to: Professor Il-Seon Park, Department of Cellular and Molecular Medicine, Chosun University, Seosuk-dong, Dong-gu, Gwangju 501-759, Republic of Korea
E-mail: parkis@chosun.ac.kr

Present address: ⁴Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

Abbreviations: A β , 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

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

The binding of A β 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 accumulation of A β 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 A β (22,23) and the conformational states of A β (oligomer vs. fibril) (12,24,25).

Although a number of studies have established a major role for apoptosis in A β -induced cell death, it is frequently observed that caspase activation is not potent in A β -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 A β 42. The inhibitory effect of A β 42 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 A β 42 interacts with other proteins involved in apoptosis and disrupts their functions, resulting in low levels of caspase activation. However, based on the studies regarding A β -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 A β 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 A β peptide. A β 42 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 A β 42 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. A β 42 oligomers were prepared by dissolution of the peptide powder in cell culture media at 100 μ M and incubation at 4°C for 12 h as previously described (29). To prepare the fibrils, the A β 42 powder at 100 μ M 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% CO₂. 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 A β 42 for the indicated periods of time or STS (0.5 μ M) 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 μ l of 5 mg/ml MTT solution in PBS, incubated for 2 h and then with 100 μ l 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 μ l 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 A β 42 preparations or other damaging agents, the cells were washed twice with ice-cold PBS. Subsequently, 40 μ l 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 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 2 μ g/ml aprotinin, 25 μ g/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 MgCl₂, 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 μ g/ml leupeptin, 2 μ g/ml pepstatin A and 2 μ g/ml aprotinin) for 20 min on ice, as previously described (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 NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, and 190 μ g/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 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 2 μ g/ml aprotinin) and the pellets were resuspended in a buffer containing 25 mM Tris-HCl (pH 8.0) and 1% Triton X-100. 30 μ g 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 μ g/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 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 2 μ g/ml aprotinin and 25 μ g/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 μ g) with 1 mM dATP, and 1-10 μ M 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 MgCl₂ 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 TGTTC 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 A β 42 induces the minimal activation of caspases-3, -8 and -9. We initially sought an experimental condition under which caspase would be robustly activated in A β -treated cells to probe the A β -induced apoptotic pathway. To achieve this, we sought to use A β -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 μ M A β 42 for up to 48 h (Fig. 1). The oligomeric preparation of A β 42 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, A β 42 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 A β 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 A β 42-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 A β -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 A β 42-induced damage, as no A β 42-concentration-dependent activity was observed.

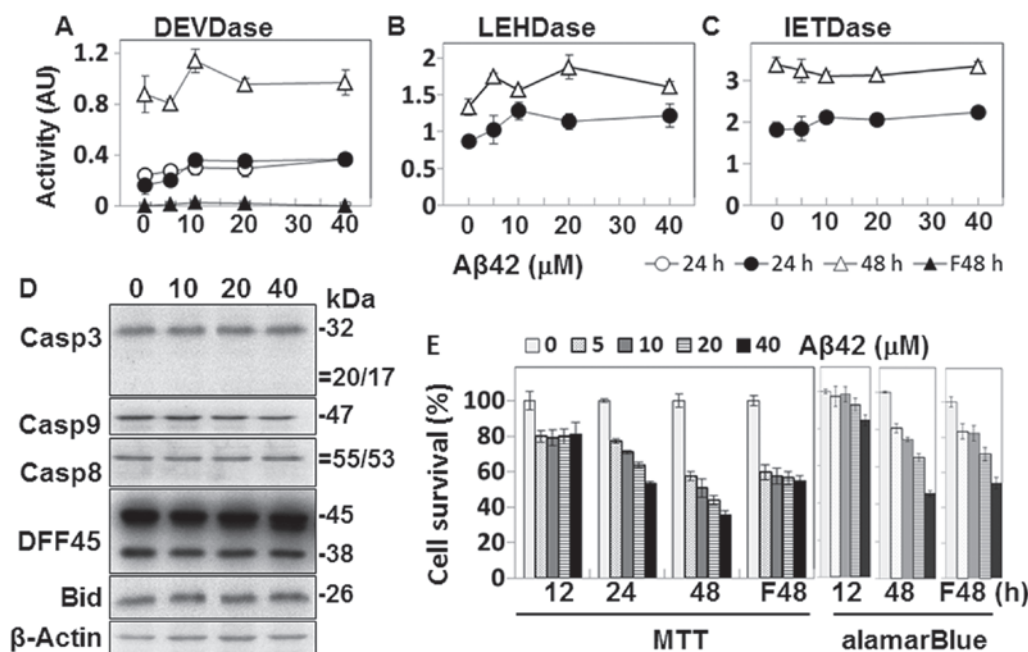


Figure 1. Activation of caspases, processing of their substrates, and cell death in amyloid- β (A β)42-singly-treated cells. (A-C) DEVDase, LEHDase, and IETDase activities were measured with 10 μ M DEVD-AMC, 50 μ M LEHD-AMC, and 50 μ M IETD-AMC for cells treated with A β 42 at the indicated concentrations and durations. AU, arbitrary unit. (D) Western blot analysis of caspases and their substrates was performed using cells treated with A β 42 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 A β 42 at the indicated concentrations and time-points using MTT reduction and alamarBlue assays. F48 indicates samples treated with fibrillary A β 42 for 48 h. The results of (A-C and E) are expressed as the means \pm 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 A β 42-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 A β 42 under the experimental conditions described above.

A β 42 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 A β 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 A β 42-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 A β 42-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 A β 42 peptide used herein was cytotoxic.

It has been reported that A β 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 A β 42-treated cells (Fig. 1A). MTT formazan production was reduced in the cells treated with the fibrillary form of A β 42, but it was not A β 42-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 A β -dose-dependent reduction was clearly observed in the alamarBlue assay on the same samples, suggesting that A β 42-dose-dependent cell death occurred (Fig. 1E). Taken together, these data suggest that the fibrillar form of A β 42 led to cell death in which caspase-dependent apoptosis played a minimal role in the process. In the following experiments, fibrillar A β 42 was not used, due to the lack of caspase activation in the peptide-treated cells (Fig. 1A).

Double treatment with A β induces potent caspase activation.

As A β 42-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 A β -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 potentially activated. After testing different time points, a 2-h initial treatment of A β 42 at the indicated concentrations was selected, as it resulted in the highest caspase activity (other time point data not shown). At

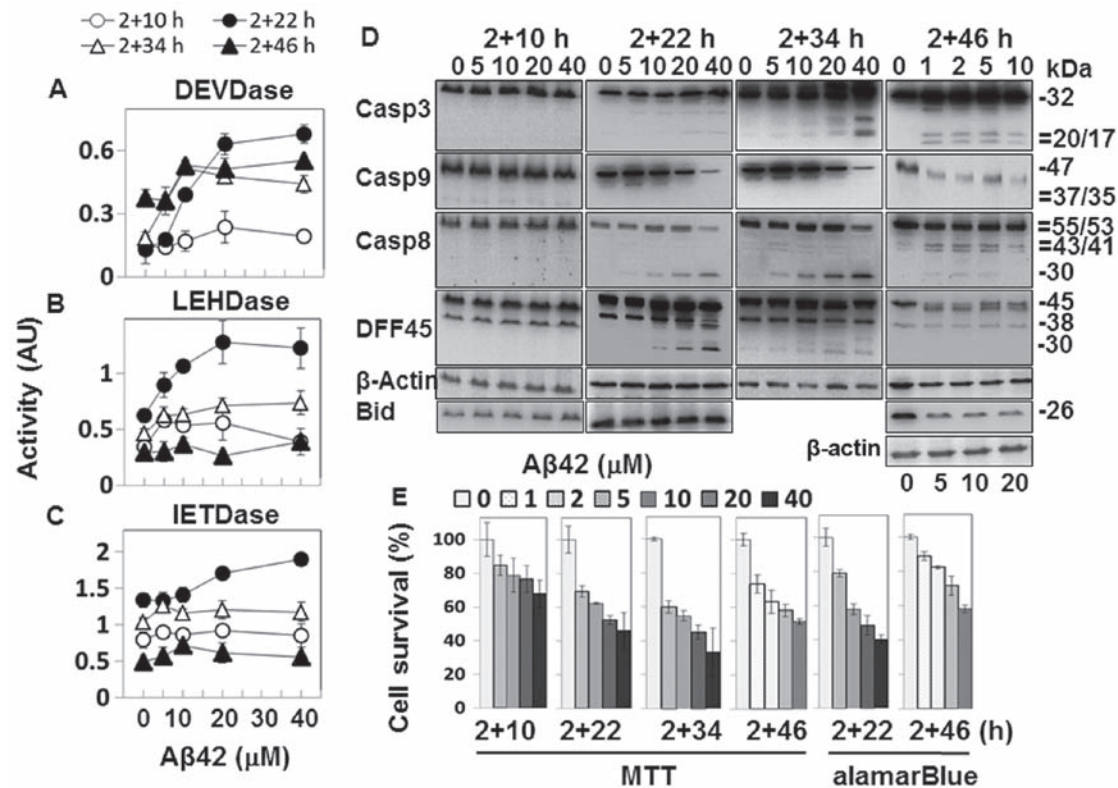


Figure 2. Activation of caspases, processing of their substrates, and cell death in amyloid- β (A β)42-doubly-treated-cells. (A-C) Enzyme activities were determined as described for Fig. 1A for cells doubly treated with A β 42 at the indicated concentrations and time. (D) Western blot analysis of caspases and their substrates was performed using cells treated with A β 42 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 A β 42 (up to 20 μ M for Bid and 10 μ M 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 A β 42 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 \pm 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 A β 42 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 A β 42 for 1 h followed by treatment with soluble A β 42; however, in this study, we used only soluble oligomeric A β 42, 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 A β 42-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 A β 42, 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 A β 42-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 dura-

tions (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 A β 42 (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 A β 42-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 matured. Thus, the cleavage sites to form the two different fragments are distinct (37).

In order to determine the cytotoxicity of the A β 42 peptide preparation, cell death was assessed in the double treatment samples. All the samples tested showed A β 42-dose-dependent cell death (Fig. 2E). The levels of cell death evaluated by MTT-formazan reduction were generally higher than those by

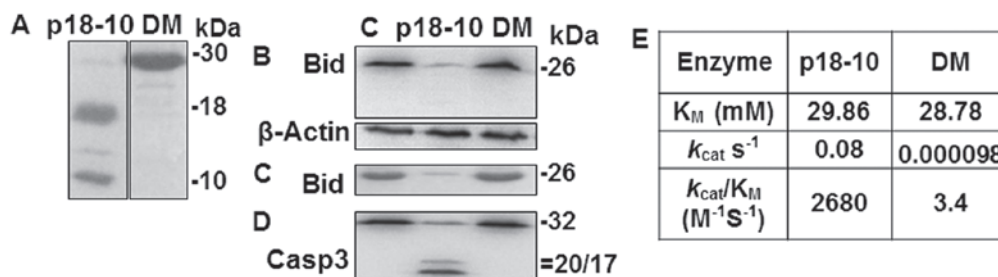


Figure 3. Activities of purified caspase-8 variants. (A) Purified p18-10 and DM are shown using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). (B and C) Processing was examined for Bid from cell extracts (70 μ g) (B) or purified Bid (1 μ g) (C) that was incubated with 30 ng of 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 A β 42 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 \sim 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 A β 42 at concentrations higher than 5 μ M (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 A β 42-doubly-treated cells. Catalytic activity and the processing of caspases-9 and -3 in the A β 42-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 μ M A β 42, 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 μ M A β 42. Apaf-1 and caspase-9 were detected only in the later fractions (>25) of SEC in the control sample prepared from A β 42-non-treated cells (Fig. 4B) or cells treated once with A β 42 for 24 h (Fig. 4C), whereas they were detected in the earlier fractions (\sim 15–23) in the sample treated with 20 μ M of A β 42 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 A β 42-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 (\sim 40% of total cytochrome *c* protein) in the 2+22 h sample treated with 20 or 40 μ M of A β 42 (Fig. 4A), compared with that of STS-treated cells (\sim 90%) (Fig. 5A). Cytochrome *c* is an essential constituent of apoptosome. Thus, the reduced level of released cytochrome *c* by A β 42 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 μ M A β 42 (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 (\sim 34% of total caspase-9 protein) of caspase-9 (\sim 40%) bound to the protein complex formed in the 2+22 h A β 42 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

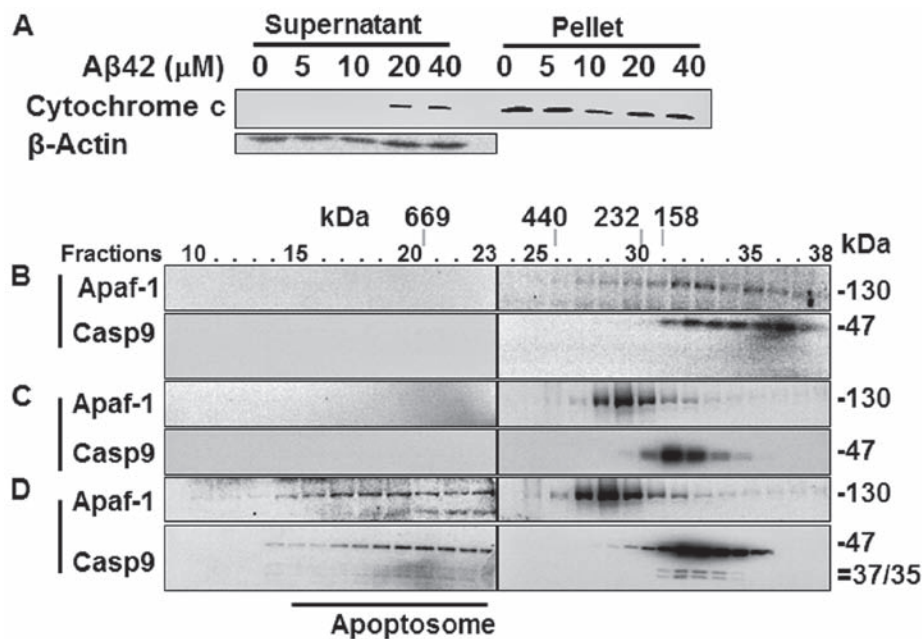


Figure 4. Cytochrome *c* release and the formation of the apoptotic protease activating factor-1 (Apaf-1) apoptosome in amyloid- β (A β)42-doubly treated cells. (A) Cytochrome *c* release was assessed by western blot analysis of soluble (supernatant) and precipitated (pellet) parts of the extracts prepared from cells treated with 20 μ M A β 42 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 μ M A β 42 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.

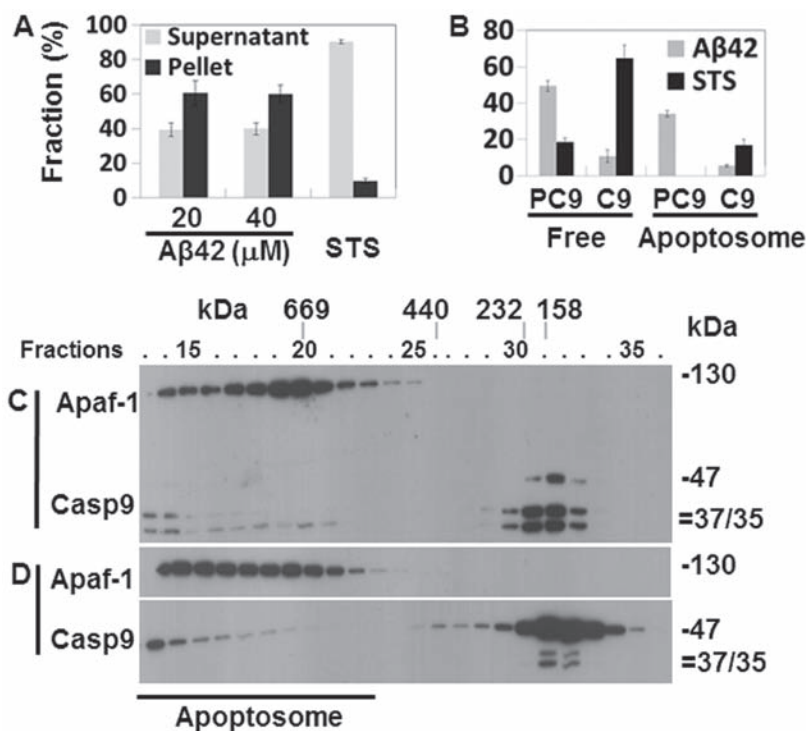


Figure 5. Comparison of released cytochrome *c* and processing of procaspase-9 in staurosporine (STS) and the 2+22 h amyloid- β (A β)42-treated samples and cell-free formation of apoptosome. (A and B) Optical densities of immunoreactive bands of cytochrome *c* and caspase-9 from Fig. 4A and D were quantified and compared with those obtained in STS-treated cells which were adopted from ref. (27). The results are expressed as the means \pm 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 μ M cytochrome *c* in the absence (C) or presence of 20 μ M A β 42 (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 relative 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 A β 42). 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),

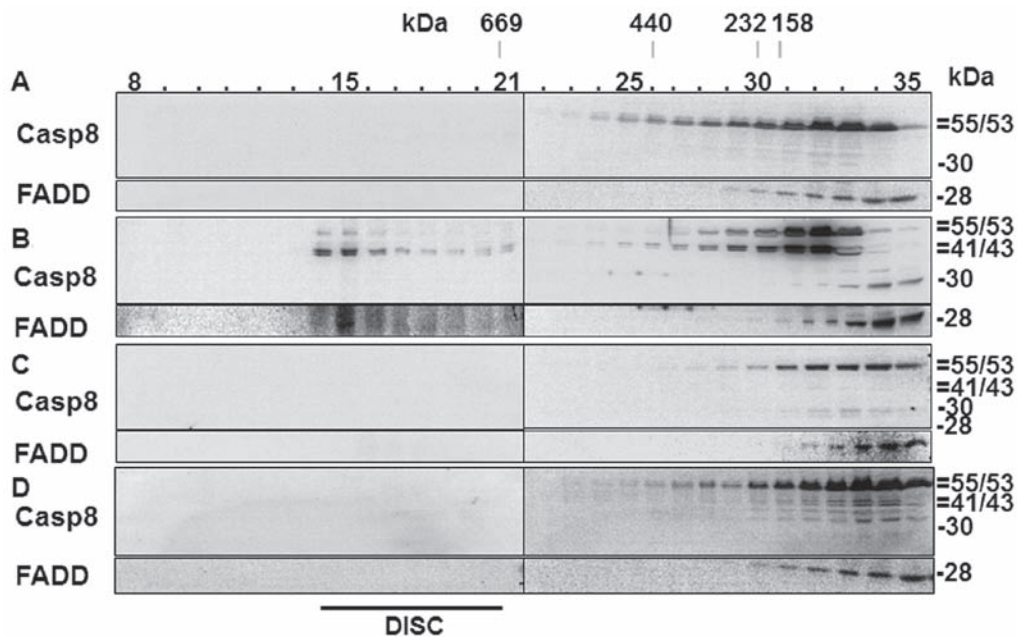


Figure 6. Absence of death-inducing signaling complex (DISC) assembly in amyloid- β (A β)42-doubly-treated cells. Cells were incubated without (A), with 100 μ g/ml of actinomycin D for 2 h and 50 nM of tumor necrosis factor- α (TNF- α) for another 24 h (B), with 40 μ M A β 42 for 2+22 h (C), or 10 μ M A β 42 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 μ M A β 42 contained only unprocessed procaspase-9 (Fig. 5D). These data clearly indicate that A β 42 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 A β 42 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 A β 42 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 A β 42-treated cells. A number of studies have indicated that EAPW plays a crucial role in the apoptotic process induced by A β 42 (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 A β 42-treated cells previously. We probed formation of DISC in the A β 42-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 μ M A β 42, 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 μ M A β 42, 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 μ M A β 42 were through a pathway other than EAPW. Higher concentrations (>20 μ M) of A β 42 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 A β 42 (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 μ M A β 42 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

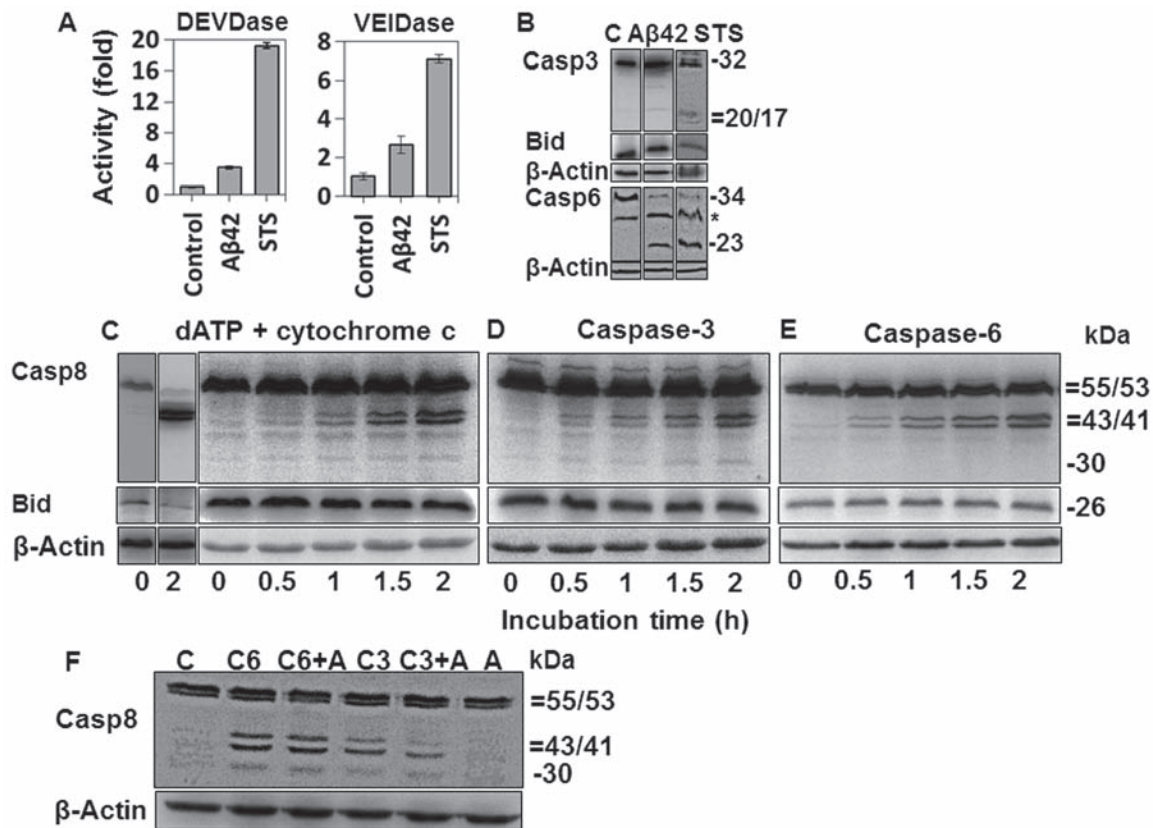


Figure 7. Activation of caspases-3 and -6 in amyloid- β (A β)42-doubly-treated or staurosporine (STS)-treated cells and fragmentation of procaspase-8 in cell-free apoptotic process. (A) Activities of caspases-3 and -6 were measured for cells treated with 20 μ M A β 42 for 2+22 h or with 0.1 mM STS for 6 h, using 10 μ M DEVD-AMC and 50 μ M VEID-AMC as substrates. Activity is indicated by fold increase over that of the control and expressed as mean \pm standard deviation of 3 independent experiments. DEVDase data of the A β 42 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 μ M for lane 2 and 1 μ M 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 μ g) were incubated with purified caspase-6 (50 ng) or caspase-3 (30 ng) in the presence or absence of 20 μ M A β 42, 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 A β 42, respectively. Casp, caspase.

approach, in which cell extracts were incubated with 1 mM dATP and 10 μ M 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 μ M) 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 incu-

bated for 2+22 h with 20-40 μ M A β 42, 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 A β 42-doubly-treated cells. However, it was noted that DEVDase and VEIDase activity, and processing of the two caspases were less in the A β 42-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 A β 42-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 A β 42 could negatively affect the activity of caspases-3 and -6 for the processing of procaspase-8. To test this hypothesis, the effect of A β 42 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 A β 42 treatment, while caspase-3 appeared to be only slightly affected by the

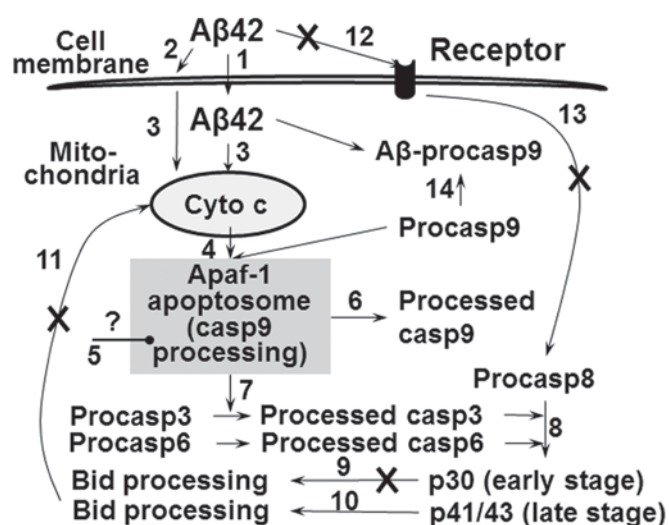


Figure 8. Summary of proposed model for the effect of A β on apoptosis pathway. Amyloid- β (A β)42 enters into cell (1), interacts with cell membrane or forms a channel-like structure on cell membrane (2) to induce intrinsic apoptosis pathway (IAPW) (3). Cytochrome *c* (cyto *c*) is released from the 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 A β 42-treated cells (12) and caspase-8 appears to be activated by IAPW (8) instead of extrinsic apoptosis pathway (EAPW) (13). A β 42 can interact with procaspase-9 to suppress the apoptosome formation (14). However, in 2+22 h sample with 20 μ M A β 42 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 A β 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 A β 42-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 A β 42-doubly-treated cells (Fig. 2A-C). This is consistent with our previous study demonstrating that A β 42 did not affect the activities of caspases except on procaspase-9 (27).

Discussion

Although caspase is not robustly activated in A β -treated cells (Figs. 1 and 6A and B) (26), and A β 42 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 A β 42 (19,49). In the present study, we sought a caspase-activating condition in A β 42-treated cells and explored the apoptosis pathways involved under the condition to assess cytotoxic properties of A β 42 and its mechanistic features. To achieve this, we explored various culture systems and methods associated with A β 42 treatment in the experiments.

Caspase activity was detected in the cells treated 'once' with A β 42 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, A β 42-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 A β 42. For example, it is possible that serum deprivation of the cells before A β 42 treatment caused increased background activity. Thus, it is not clear whether the activity was due to A β 42 treatment or other unknown factors.

The activation of caspase was observed in cells 'doubly' treated with A β 42 in which A β 42-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 A β 42-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 A β 42 to induce the activation of caspases remains poorly understood. The suppressive effect of A β 42 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 A β 42 might be an inducing factor for the formation of apoptosome.

However, A β 42-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 A β 42 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 A β 42 (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 A β 42 and STS-induced IAPW than binding of the protein to the Apaf-1 apoptosome.

Some studies have indicated that EAPW is elicited in A β -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 A β -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 μ M A β 42, 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 μ M A β 42, 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 A β 42, at higher concentrations. However, the role of the hypothetical DISC formation and EAPW activation in A β 42-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 μ M A β 42 (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 A β -induced apoptosis occurred to a significant extent. Therefore, it is questionable whether they played active roles in the A β -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 A β 42-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 A β 42 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 A β 42-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 A β 42-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 A β 42 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 A β 42-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 A β 42 on apoptosis pathway was summarized in Fig. 8.

A previous study showed that the double treatment of A β 42 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 A β 42 (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 A β 42-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 A β 42 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 A β -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 A β -associated pathology and control of AD.

Acknowledgments

This study was partially supported by funding from the Korea Research Foundation Grant from the Korean Government (no. K201474026) and the Chosun University research fund in 2014. MII is a member of Korean Government Scholarship Program-2010 (Graduate stream).

References

- Esler WP and Wolfe MS: A portrait of Alzheimer secretases - new features and familiar faces. *Science* 293: 1449-1454, 2001.
- Mattson MP: Pathways towards and away from Alzheimer's disease. *Nature* 430: 631-639, 2004.
- Hardy J and Selkoe DJ: The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science* 297: 353-356, 2002.
- Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW and Glabe CG: Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300: 486-489, 2003.
- Neudecker P, Robustelli P, Cavalli A, Walsh P, Lundström P, Zarrine-Afsar A, Sharpe S, Vendruscolo M and Kay LE: Structure of an intermediate state in protein folding and aggregation. *Science* 336: 362-366, 2012.
- Selkoe DJ: American College of Physicians; American Physiological Society: Alzheimer disease: Mechanistic understanding predicts novel therapies. *Ann Intern Med* 140: 627-638, 2004.
- Duyckaerts C, Delatour B and Potier MC: Classification and basic pathology of Alzheimer disease. *Acta Neuropathol* 118: 5-36, 2009.
- Walsh DM, Minogue AM, Sala Frigerio C, Fadeeva JV, Wasco W and Selkoe DJ: The APP family of proteins: Similarities and differences. *Biochem Soc Trans* 35: 416-420, 2007.
- Chong YH, Shin YJ, Lee EO, Kaye R, Glabe CG and Tenner AJ: ERK1/2 activation mediates Abeta oligomer-induced neurotoxicity via caspase-3 activation and tau cleavage in rat organotypic hippocampal slice cultures. *J Biol Chem* 281: 20315-20325, 2006.
- Ellerby LM and Orr HT: Neurodegenerative disease: Cut to the chase. *Nature* 442: 641-642, 2006.
- Nikolaev A, McLaughlin T, O'Leary DD and Tessier-Lavigne M: APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature* 457: 981-989, 2009.
- Picone P, Carrotta R, Montana G, Nobile MR, San Biagio PL and Di Carlo M: Abeta oligomers and fibrillar aggregates induce different apoptotic pathways in LAN5 neuroblastoma cell cultures. *Biophys J* 96: 4200-4211, 2009.
- Fossati S, Ghiso J and Rostagno A: TRAIL death receptors DR4 and DR5 mediate cerebral microvascular endothelial cell apoptosis induced by oligomeric Alzheimer's A β . *Cell Death Dis* 3: e321, 2012.

14. Fossati S, Ghiso J and Rostagno A: Insights into caspase-mediated apoptotic pathways induced by amyloid- β in cerebral microvascular endothelial cells. *Neurodegener Dis* 10: 324-328, 2012.
15. Boatright KM and Salvesen GS: Mechanisms of caspase activation. *Curr Opin Cell Biol* 15: 725-731, 2003.
16. Mace PD, Riedl SJ and Salvesen GS: Caspase enzymology and activation mechanisms. *Methods Enzymol* 544: 161-178, 2014.
17. Troy CM, Friedman JE and Friedman WJ: Mechanisms of p75-mediated death of hippocampal neurons. Role of caspases. *J Biol Chem* 277: 34295-34302, 2002.
18. Truzzi F, Marconi A, Atzei P, Panza MC, Lotti R, Dallaglio K, Tiberio R, Palazzo E, Vaschieri C and Pincelli C: p75 neurotrophin receptor mediates apoptosis in transit-amplifying cells and its overexpression restores cell death in psoriatic keratinocytes. *Cell Death Differ* 18: 948-958, 2011.
19. Cha MY, Han SH, Son SM, Hong HS, Choi YJ, Byun J and Mook-Jung I: Mitochondria-specific accumulation of amyloid β induces mitochondrial dysfunction leading to apoptotic cell death. *PLoS One* 7: e34929, 2012.
20. Murakami Y, Ohsawa I, Kasahara T and Ohta S: Cytoprotective role of mitochondrial amyloid beta peptide-binding alcohol dehydrogenase against a cytotoxic aldehyde. *Neurobiol Aging* 30: 325-329, 2009.
21. Lee DY, Lee KS, Lee HJ, Kim DH, Noh YH, Yu K, Jung HY, Lee SH, Lee JY, Youn YC, *et al*: Activation of PERK signaling attenuates Abeta-mediated ER stress. *PLoS One* 5: e10489, 2010.
22. Verdier Y and Penke B: Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. *Curr Protein Pept Sci* 5: 19-31, 2004.
23. Verdier Y, Zaránde M and Penke B: Amyloid beta-peptide interactions with neuronal and glial cell plasma membrane: Binding sites and implications for Alzheimer's disease. *J Pept Sci* 10: 229-248, 2004.
24. Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA and LaDu MJ: Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J Biol Chem* 277: 32046-32053, 2002.
25. Resende R, Ferreiro E, Pereira C and Resende de Oliveira C: Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: Involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. *Neuroscience* 155: 725-737, 2008.
26. Thapa A, Woo ER, Chi EY, Sharoar MG, Jin HG, Shin SY and Park IS: Biflavonoids are superior to monoflavonoids in inhibiting amyloid- β toxicity and fibrillogenesis via accumulation of nontoxic oligomer-like structures. *Biochemistry* 50: 2445-2455, 2011.
27. Sharoar MG, Islam MI, Shah Nawaz M, Shin SY and Park IS: Amyloid β binds procaspase-9 to inhibit assembly of Apaf-1 apoptosome and intrinsic apoptosis pathway. *Biochim Biophys Acta* 1843: 685-693, 2014.
28. Thapa A, Shah Nawaz M, Karki P, Raj Dahal G, Sharoar MG, Yub Shin S, Sup Lee J, Cho B and Park IS: Purification of inclusion body-forming peptides and proteins in soluble form by fusion to *Escherichia coli* thermostable proteins. *Biotechniques* 44: 787-796, 2008.
29. Chromy BA, Nowak RJ, Lambert MP, Viola KL, Chang L, Velasco PT, Jones BW, Fernandez SJ, Lacor PN, Horowitz P, *et al*: Self-assembly of Abeta(1-42) into globular neurotoxins. *Biochemistry* 42: 12749-12760, 2003.
30. Karki P, Dahal GR and Park IS: Both dimerization and interdomain processing are essential for caspase-4 activation. *Biochem Biophys Res Commun* 356: 1056-1061, 2007.
31. Wogulis M, Wright S, Cunningham D, Chilcote T, Powell K and Rydel RE: Nucleation-dependent polymerization is an essential component of amyloid-mediated neuronal cell death. *J Neurosci* 25: 1071-1080, 2005.
32. Slaughter MR, Bugelski PJ and O'Brien PJ: Evaluation of alamar blue reduction for the in vitro assay of hepatocyte toxicity. *Toxicol In Vitro* 13: 567-569, 1999.
33. Karki P, Seong C, Kim JE, Hur K, Shin SY, Lee JS, Cho B and Park IS: Intracellular K(+) inhibits apoptosis by suppressing the Apaf-1 apoptosome formation and subsequent downstream pathways but not cytochrome *c* release. *Cell Death Differ* 14: 2068-2075, 2007.
34. Majkut J, Sgobba M, Holohan C, Crawford N, Logan AE, Kerr E, Higgins CA, Redmond KL, Riley JS, Stasik I, *et al*: Differential affinity of FLIP and procaspase 8 for FADD's DED binding surfaces regulates DISC assembly. *Nat Commun* 5: 3350, 2014.
35. Estus S, Tucker HM, van Rooyen C, Wright S, Brigham EF, Wogulis M and Rydel RE: Aggregated amyloid-beta protein induces cortical neuronal apoptosis and concomitant 'apoptotic' pattern of gene induction. *J Neurosci* 17: 7736-7745, 1997.
36. Xue WF, Homans SW and Radford SE: Systematic analysis of nucleation-dependent polymerization reveals new insights into the mechanism of amyloid self-assembly. *Proc Natl Acad Sci USA* 105: 8926-8931, 2008.
37. Hoffmann JC, Pappa A, Krammer PH and Lavrik IN: A new C-terminal cleavage product of procaspase-8, p30, defines an alternative pathway of procaspase-8 activation. *Mol Cell Biol* 29: 4431-4440, 2009.
38. Li H, Zhu H, Xu CJ and Yuan J: Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94: 491-501, 1998.
39. Oberst A, Pop C, Tremblay AG, Blais V, Denault JB, Salvesen GS and Green DR: Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. *J Biol Chem* 285: 16632-16642, 2010.
40. Pop C, Oberst A, Drag M, Van Raam BJ, Riedl SJ, Green DR and Salvesen GS: FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. *Biochem J* 433: 447-457, 2011.
41. Pop C, Fitzgerald P, Green DR and Salvesen GS: Role of proteolysis in caspase-8 activation and stabilization. *Biochemistry* 46: 4398-4407, 2007.
42. Salvesen GS and Dixit VM: Caspase activation: The induced-proximity model. *Proc Natl Acad Sci USA* 96: 10964-10967, 1999.
43. Beaudouin J, Liesche C, Aschenbrenner S, Hörner M and Eils R: Caspase-8 cleaves its substrates from the plasma membrane upon CD95-induced apoptosis. *Cell Death Differ* 20: 599-610, 2013.
44. Qian MC, Liu J, Yao JS, Wang WM, Yang JH, Wei LL, Shen YD and Chen W: Caspase-8 mediates amyloid- β -induced apoptosis in differentiated PC12 cells. *J Mol Neurosci* 56: 491-499, 2015.
45. Hippe D, Lytovchenko O, Schmitz I and Lüder CG: Fas/CD95-mediated apoptosis of type II cells is blocked by Toxoplasma gondii primarily via interference with the mitochondrial amplification loop. *Infect Immun* 76: 2905-2912, 2008.
46. Cowling V and Downward J: Caspase-6 is the direct activator of caspase-8 in the cytochrome *c*-induced apoptosis pathway: Absolute requirement for removal of caspase-6 prodomain. *Cell Death Differ* 9: 1046-1056, 2002.
47. Monnier PP, D'Onofrio PM, Magharious M, Hollander AC, Tassew N, Szydlowska K, Tymianski M and Koeberle PD: Involvement of caspase-6 and caspase-8 in neuronal apoptosis and the regenerative failure of injured retinal ganglion cells. *J Neurosci* 31: 10494-10505, 2011.
48. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, *et al*: Ordering the cytochrome *c*-initiated caspase cascade: Hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 144: 281-292, 1999.
49. Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, Davis RJ, Shirasaki Y and Greenberg ME: Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. *J Neurosci* 21: 7551-7560, 2001.
50. Jan A, Adolfsson O, Allaman I, Buccarello AL, Magistretti PJ, Pfeifer A, Muhs A and Lashuel HA: Abeta42 neurotoxicity is mediated by ongoing nucleated polymerization process rather than by discrete Abeta42 species. *J Biol Chem* 286: 8585-8596, 2011.
51. Wiedert T, Essmann F, Prokop A, Schmelz K, Schulze-Osthoff K, Beyaert R, Dörken B and Daniel PT: Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. *Blood* 97: 1378-1387, 2001.