Amyloid Precursor Protein Binding Protein Fe65 Is Cleaved by Caspases during DNA Damage-Induced Apoptosis

Kazunori SAEKI,^a Yasuyo NOSE,^a Nobukuni HIRAO,^a Ryoko TAKASAWA,^a and Sei-ichi TANUMA^{*,a,b}

^a Department of Biochemistry, Faculty of Pharmaceutical Science, Tokyo University of Science; and ^b Genome and Drug Research Center, Tokyo University of Science; 2641 Yamazaki, Noda, Chiba 278–8510, Japan.

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Caspases cleave several cellular proteins to execute cell death by apoptosis. The identification of novel substrates of caspases could provide an important clue for elucidation of new apoptosis signaling pathways. In this study, we tested whether an amyloid precursor protein (APP) binding protein Fe65 is proteolytically degraded in neuronal cell death by apoptosis, using a neuron-like cell line, human neuroblastoma SH-SY5Y cells. When treated with DNA damaging agents, etoposide (ETP) and camptothecin (CPT), SH-SY5Y cells underwent apoptosis in a dose-dependent manner. Interestingly, Fe65 (97 kDa) was cleaved to a 65 kDa product during DNA damage-induced apoptosis. Furthermore, the cleavage of Fe65 was accompanied by activation of caspases-9 and -3. The restriction cleavage of Fe65 was completely suppressed by the treatment with a pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethylketone (z-VAD-fmk). These results reveal the restriction cleavage of Fe65 by caspases during DNA damage-induced apoptosis. Since Fe65 has been shown to suppress APP processing to amyloid β ($A\beta$) production, our findings may provide a new insight into the molecular mechanism by which DNA damage induces $A\beta$ production and subsequent neuronal cell death in Alzheimer's disease (AD).

Key words caspase; apoptosis; DNA damage; Fe65; Alzheimer's disease; amyloid precursor protein

Caspases are a class of cysteine proteases which play a key role in apoptosis.¹⁾ The caspases convey apoptotic signals in proteolytic cascades; executioner caspases activated by initiator caspases degrade cellular protein substrates that lead to cell death, such as poly(ADP-ribose) polymerase-1 (PARP-1), inhibitor of CAD (ICAD) and Bid.^{2–4)} Recently, we have identified checkpoint kinase 1 (Chk1), which plays important roles in cell cycle checkpoints during cell cycle progression, as a novel substrate cleaved in a caspase-dependent manner during DNA damage-induced apoptosis.⁵⁾ The finding of the restriction cleavage of Chk1 by caspases during apoptosis reveals the presence of a novel regulatory mechanism for apoptotic commitment. It is possible that unidentified substrates of caspases have important roles in the determination of cell fate, death or survival. Thus, the identification of novel substrates of caspases could provide a clue for elucidation of new apoptosis signaling pathways. We show here, for the first time, that an amyloid precursor protein (APP) binding protein, Fe65, is cleaved by caspases during DNA damage-induced apoptosis in a neuron-like cell line, SH-SY5Y cells derived from human neuroblastoma.

Fe65 is an adaptor protein mediating assembly of multi-molecular complexes through a variety of protein interaction domains (WW domain, phosphotyrosine binding (PTB) 1 domain and PTB2 domain).^{6–8)} Interestingly, Fe65 has been shown to interact with APP through the PTB2 domain and modulate the processing of APP, and thereby reduce amyloid β ($A\beta$) production.^{9–11)} It is generally believed that abnormal production and aggregation of $A\beta$ are primary pathogenic events leading to neuronal cell death in Alzheimer's disease (AD).^{12–14)} Since Fe65 is involved in the regulation of normal APP processing for cell survival,^{9–11)} it seems to be reasonable that apoptotic caspases cleave and inactivate survival regulatory factors, such as Fe65, for the efficient progression of neuronal cell death after DNA damage.

It has been reported that DNA damage is closely involved in AD. There is a lot of evidence that brain tissue in AD

patients is exposed to DNA damage during the course of the disease.¹⁵⁾ Evidence of DNA damage in AD is the elevated level of oxidized DNA bases in mitochondrial and nuclear DNA.^{16,17)} Furthermore, it has been shown that DNA damaging agents activate γ -secretase and elevate $A\beta$ production followed by neuronal cell death.^{18,19)} However, although $A\beta$ is generated through the sequential cleavage of APP by β - and γ -secretases, no details are available concerning the mechanism underlying the regulation of $A\beta$ production during DNA damage-induced apoptosis. Thus, for the understanding of the regulatory mechanism of $A\beta$ production, the identification of closely related events and molecules is necessary. One of the interesting questions is whether molecular modulation of Fe65 occurs during DNA damage-induced apoptosis.

In this study, we found that Fe65 is proteolytically cleaved during apoptosis induced by DNA damaging agents in SH-SY5Y cells. Furthermore, this cleavage is a caspase dependent manner, suggesting that intrinsic apoptotic pathway elicited by DNA damaging agents could modulate the processing of APP to $A\beta$ production through the restriction cleavage of Fe65. Our data indicate that the caspase-mediated cleavage of Fe65 may diminish its suppression function to $A\beta$ production and that it may be a mechanism by which the production of $A\beta$ is enhanced. Such information may shed new lights on how DNA damage causes neuronal cell death by apoptosis in AD.

MATERIALS AND METHODS

Materials Etoposide (ETP) and camptothecin (CPT) were purchased from WAKO (Osaka, Japan). *N*-Benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethylketone (z-VAD-fmk) were from Peptide Institute. Mouse anti-Fe65 monoclonal antibody (clone 324H) was from Abcam (Cambridge, U.K.). Mouse anti- β -actin monoclonal antibody (clone AC-15) and mouse anti-PARP-1 monoclonal antibody (clone C2-

* To whom correspondence should be addressed. e-mail: tanuma@rs.noda.tus.ac.jp

Fig. 1. Induction of Apoptosis by Etoposide (ETP) and Camptothecin (CPT) in SH-SY5Y Cells

SH-SY5Y cells were treated with indicated concentrations of ETP and CPT for 12 h and then harvested. (A) The appearance of apoptotic cells by the treatment with ETP (upper) or CPT (lower) was analyzed by morphological examination as described under Materials and Methods. (B) The cleavage of PARP-1 was analyzed by Western blot as described under Materials and Methods. β -Actin is for loading control.

10) were from SIGMA and WAKO, respectively. Rabbit anti-caspase-3 polyclonal antibody and rabbit anti-caspase-9 polyclonal antibody were from Cell Signaling Technology. Goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugate and goat anti-rabbit IgG HRP-conjugate were from Jackson Immunological Research.

Cell Culture A neuron-like cell line human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μ g/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

Western Blot Analysis Harvested cell samples were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol and 0.1% bromophenol blue). Cell lysates were separated by SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were blocked with 2.5% skim milk and 0.25% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 1 h at room temperature, and then probed with appropriate primary antibodies for overnight at 4 °C. After the membranes were washed with TTBS, incubated with the appropriate secondary antibody for 1 h at room temperature. After washing the membrane with TTBS, the blotted proteins were visualized with Light Capture System (ATTO, Tokyo, Japan) by using Supersignal West Femto Maximum Sensitivity Substrate (PIERCE, Rockford, IL, U.S.A.).

Detection of Apoptosis Apoptosis was induced by DNA damaging agents, ETP and CPT. Cells were collected for morphological examination. After fixed with 1% (w/v) glutaraldehyde, the frequencies of apoptotic cells were determined by fluorescence microscopy with Hoechst 33258 staining. Cells with condensed chromatin or fragmented nuclei were counted as apoptotic cells.²⁰⁾

RESULTS

Fe65 Is Cleaved during DNA Damage-Induced Apoptosis A neuron-like cell line, human neuroblastoma SH-SY5Y cells constitutively express an APP binding protein, Fe65.²¹⁾ Using this cell line, we examined whether the structure and function of Fe65 are modulated during apoptosis induced by DNA damaging agents, ETP and CPT.

Initially, we investigated the apoptosis-inducibilities of ETP and CPT in SH-SY5Y cells. As shown in Fig. 1A, ETP induced apoptosis in SH-SY5Y cells in a dose-dependent manner. Treatment with CPT, another DNA damaging agent, also induced apoptosis in a dose-dependent manner (Fig. 1A). The EC₅₀ values of ETP and CPT for apoptosis induction were approximately 5 μ M and 80 nM, respectively. Furthermore, the cleaved product of PARP-1 (85 kDa) probably catalyzed by caspase-3, which is a hallmark of apoptosis, was observed by the treatment with ETP or CPT in a dose-dependent manner (Fig. 1B). This restriction cleavage was almost consistent with the degree of apoptotic morphological changes (Fig. 1A). These results implicate that SH-SY5Y cells undergo apoptosis by the treatment with DNA damaging agents. Since ETP and CPT have been reported to induce apoptosis through mitochondria-mediated apoptosis pathway (intrinsic pathway) initiated by caspase-9,²²⁾ it is suggested that ETP and CPT induce apoptosis in SH-SY5Y cells through intrinsic pathway initiated by caspase-9.

We next tested whether Fe65 is cleaved during DNA damage-induced apoptosis in SH-SY5Y cells. As shown in Fig. 2, in both ETP and CPT treatments, Fe65 was cleaved to a 65 kDa product in a dose-dependent manner. The dose-dependencies were consistent with those of the apoptotic morphological changes and the restriction cleavage of PARP-1 (Fig. 1). These results suggest that the cleavage of Fe65 is associated with apoptosis induced by DNA damaging agents.

The Cleavage of Fe65 Correlates with Activation of

Caspases-9 and -3 Since DNA damage is known to induce apoptosis *via* intrinsic pathway in which caspases-9 and -3 are mainly activated,²²⁾ we hypothesize that Fe65 is cleaved by these caspases like PARP-1. In intrinsic pathway, pro-caspase-9 (47 kDa) is initially cleaved to the small fragment (10 kDa) and large active fragments (17, 35, 37 kDa) in apoptosome,²³⁾ and then activated caspase-9 cleaves pro-caspase-3 (35 kDa) to the small active fragment (12 kDa) and large active fragments (17, 19 kDa).²⁴⁾

To test our hypothesis, activation of caspases-9 and -3 was analyzed by Western blot using anti-caspases-9 and -3 antibodies. The cleaved active fragments of caspases-9 and -3 increased by the treatment with ETP or CPT in a dose-dependent manner (Fig. 2). In both ETP and CPT treatment, the cleavage of Fe65 was in close association with the activation of caspases-9 and -3. In the CPT treatment, the cleavage of Fe65 reached a plateau at 100–1000 nM, while in the ETP treatment, the cleaved Fe65 was maximal at 3 μ M and then further degraded. The reason of these different degradation profiles is not yet clear. Probably, in the higher concentrations (10, 30 μ M) of ETP treatment, the cleaved Fe65 is degraded secondary by other proteases activated in the conditions. On the other hand, caspase-8, which is activated through death receptor/ligand mediated apoptosis pathway (extrinsic pathway), was not cleaved to the active fragments by the treatment with ETP or CPT (data not shown). These results support our hypothesis that Fe65 is cleaved by caspases-9 and -3 participating in intrinsic pathway.

The Cleavage of Fe65 Is Dependent on Caspase Activation We next examined the effects of caspase inhibitors on the cleavage of Fe65 induced by DNA damaging agent treatment. Although specific inhibitors of individual caspase are powerful tools for identification of responsible caspases that cleave Fe65, there is unfortunately no available specific inhibitors. Indeed, a well known caspase-3 inhibitor, DEVD-fmk, inhibits other caspases-7, -8 and -9. Also, commercially available tetrapeptide inhibitors, IETD-fmk and LEHD-fmk, for caspases-8 and -9, respectively, have no clear specificity. Furthermore, these tetrapeptide inhibitors are hard to penetrate plasma membranes.

Thus, we used an irreversible and cell permeable pan-caspase inhibitor, z-VAD-fmk, to investigate the caspase-dependent cleavage of Fe65. As shown in Fig. 3, z-VAD-fmk inhibited the cleavage of Fe65 induced by ETP and CPT in a dose-dependent manner. Also, the restriction cleavage of

Fig. 2. The Cleavage of Fe65 in SH-SY5Y Cells by the Treatment of ETP or CPT

SH-SY5Y cells were treated with indicated concentrations of ETP (A) or CPT (B) for 12 h and then harvested. To determine the cleavage of Fe65, harvested cells were lysed and analyzed by Western blot as described under Materials and Methods.

Fig. 3. Effect of a Pan-Caspase Inhibitor z-VAD-fmk on the Cleavage of Fe65 Induced by ETP or CPT Treatment

SH-SY5Y cells were pretreated with z-VAD-fmk at the indicated concentrations for 1 h and then treated with ETP (30 μ M) or CPT (1 μ M) for another 12 h. The cells were harvested and the cleavage of Fe65 (A) and PARP-1 (B) were analyzed by Western blot as described under Materials and Methods. β -Actin is for loading control (C).

PARP-1 was inhibited by z-VAD-fmk in the same dose-dependent manner. These results clearly show that Fe65 is a novel caspase substrate cleaved during DNA damage-induced apoptosis.

DISCUSSION

In this study, we showed, for the first time, that an APP binding protein Fe65 is cleaved by caspases during DNA damage-induced apoptosis in a neuron-like cell line, SH-SY5Y cells derived from human neuroblastoma. This finding suggests that Fe65 is a target of apoptotic caspases and provides a new concept that caspases promote neuronal cell death by not only activating pro-apoptotic molecules but also by inactivating molecules suppressing neuronal cell death, such as Fe65.

The cleavage of Fe65 was found to increase by the treatment with ETP or CPT in a dose-dependent manner during apoptosis (Figs. 1 and 2). Also, the cleavage of Fe65 was accompanied by activation of caspases-9 and -3, which is a well-known phenomenon in intrinsic apoptotic pathway (Fig. 2). Furthermore, we showed that the cleavage of Fe65 induced by ETP or CPT treatment was completely suppressed by the pan-caspase inhibitor, z-VAD-fmk (Fig. 3). Therefore, caspases-9 and -3 are candidates for the proteases catalyzing the cleavage of Fe65. Among caspases, caspase-9 is a key initiator caspase of the intrinsic apoptotic pathway.^{22,23} However, until now, there are no cell-permeable inhibitors specific for caspase-9. Furthermore, inhibition of caspase-9 results in suppression of down-stream caspase activation cascades. Also, there is no specific cell-permeable inhibitor for main executioner caspase-3. Therefore, *in vitro* cleavage assay using individual recombinant caspases and knock-down experiments are needed to identify responsible caspases catalyzing the cleavage of Fe65. We are now undergoing these experiments.

Recently, Fe65 is studied in the context of AD.⁸ Fe65 has been shown to interact with APP through the PTB2 domain and modulate the processing of APP, to reduce A β production.⁹ Overexpression of Fe65 in APP transgenic mice has been reported to result in lower cerebral A β levels.^{10,11} Thus, it is possible that the cleavage of Fe65 by caspases during DNA damage-induced apoptosis may impair the interaction between Fe65 and APP, and increase A β production (Fig. 4). It is generally believed that mismetabolism of APP, and abnormal production, aggregation and deposition of A β are central to the pathogenesis of AD.^{12–14} Recently, there is accumulating evidence that neuronal cells in AD brains receive DNA damage.¹⁵ Indeed, markers for DNA damage, such as 8-hydroxyguanine, 8-hydroxyadenine and 5-hydroxycytosine, have been largely observed in AD subjects.^{16,17} However, the relationship between DNA damage and A β production is not yet characterized. The restriction cleavage of Fe65 by caspases may be the missing link between DNA damage and A β production (Fig. 4).

To our knowledge, this is the first study to demonstrate the cleavage of Fe65 in a caspase-dependent manner during apoptosis. In conclusion, we suggest that the restriction cleavage of Fe65 by caspases may be critical in suppressing the regulatory function of Fe65 for normal APP processing and that the onset of caspase-mediated cleavage of Fe65 may

Fig. 4. The Missing Link between DNA Damage and A β Production

The schema of events after DNA damage that may contribute to neuronal cell death in AD and the regulatory role of Fe65 cleavage by caspases in A β production are illustrated.

be a commitment mechanism by which the production of A β is enhanced. Furthermore, our findings may indicate a novel apoptosis signaling pathway through which DNA damage induces neuronal cell death in AD *via* up-regulation of A β production by the cleavage of Fe65 (Fig. 4). Further studies using transgenic mice expressing Fe65 resistant to caspases and AD model mice would be crucial to prove our proposal.

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