

Activation of caspase-8 in 3-deazaadenosine-induced apoptosis of U-937 cells occurs downstream of caspase-3 and caspase-9 without Fas receptor-ligand interaction

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Abbreviations: DZA, 3-deazaadenosine; FADD, Fas-associated death domain; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; Ado-Hcy, S-adenosylhomocysteine; DZA-Hcy, 3-deazaadenosylhomocysteine; BA, bongkrekic acid; CsA, cyclosporin A; MPTP, mitochondria permeability transition pore; ANT, adenine nucleotide translocator; VDAC, voltage-dependent anion channel

Abstract

3-Deazaadenosine (DZA), a cellular methylation blocker was reported to induce the caspase-3-like activities-dependent apoptosis in U-937 cells. In this study, we analyzed the activation pathway of the caspase cascade involved in the DZA-induced apoptosis using specific inhibitors of caspases. In the U-937 cells treated with DZA, cytochrome c release from mitochondria and subsequent activation of caspase-9, -8 and -3 were observed before the induction of apoptosis. zDEVD-Fmk, a specific inhibitor of caspase-3, and zLEHD-Fmk, a specific inhibitor of caspase-9, prevented the activation of caspase-8 but neither caspase-3 nor caspase-9, indicating that caspase-8 is downstream of both caspase-3 and caspase-9, which are activated by independent pathways. zVAD-Fmk, a universal inhibitor of caspases, kept the caspase-3 from being activated but not caspase-9. Moreover, ZB4, an antagonistic Fas-antibody, exerted no effect on the activation of caspase-8 and induction of apoptosis by DZA. In addition, zVAD-Fmk and mitochondrial permeability transition pore (MPTP) inhibitors such as cyclosporin A (CsA) and bongkrekic acid (BA) did not

block the release of cytochrome c from mitochondria. Taken together, these results suggest that in the DZA-induced apoptosis, caspase-8 may serve as an executioner caspase and be activated downstream of both caspase-3 and caspase-9, independently of Fas receptor-ligand interaction. And caspase-3 seems to be activated by other caspases including IETDase-like enzyme and caspase-9 seems to be activated by cytochrome c released from mitochondria without the involvement of caspases and CsA- and BA- inhibitory MPTP.

Keywords: 3-deazaadenosine, caspase, caspase inhibitor, apoptosis

Introduction

Apoptosis, a physiological process of cell death, is critical for the normal development and function of multicellular organisms (Andreas *et al.*, 2000). Apoptosis is characterized by morphological changes including chromatin condensation, cytoplasmic shrinkage, and plasma membrane blebbing and biochemical changes including internucleosomal DNA cleavage (Kerr *et al.*, 1972; Wyllie *et al.*, 1980) and randomization of the distribution of phosphatidylserine (PS) between the inner and outer leaflets of the plasma membrane (Fadok and Henson, 1998). These changes are elicited by a broad range of physiological or experimentally applied death stimuli such as DNA damage, UV radiation, ionizing radiation and oxidative stress, and are observed in cells from diverse tissue types and species (Vaux and Strasser, 1996).

Caspase is known to be essential for apoptosis in a variety of species and models (Kumar and Lavin, 1996). Because caspase activities can be detected in all cells undergoing apoptosis, regardless of their origin or death stimuli, and they bring about most of visible changes that characterize apoptotic cell death, caspases can be thought as the central executioners of apoptosis (Cohen, 1997). Indeed, eliminating caspase activity, either by mutation or by the use of small pharmacological inhibitors, slowed down or even prevented apoptosis (Earnshaw *et al.*, 1999). Caspases are synthesized as enzymatically inert zymogens and need to be cleaved autocatalytically or by other caspases to be activated (Stennicke and Salvesen, 1998). Caspases are divided into subfamilies based on their substrate preference, extent of sequence identity, and structural similarities.

With a view of their functions, caspases can be classified into cytokine activators such as caspase-1, -4, and -5, apoptotic initiators such as caspase-8, -9, -10, and -2, and apoptotic executioners such as caspase-3, -6, and -7. Initiator caspases can activate executioner caspases, which in turn cleave diverse cellular substrates including gelsolin, fodrin, poly (ADP-ribose) polymerase (PARP), and ICAD/DFF45 resulting in characteristic changes of apoptosis. Thus, it is thought that there is a hierarchical relation between initiator caspases and executioner caspases (Cohen, 1997). The hierarchical model of caspase activation was well established in the two principal pathways of apoptosis induced by Fas receptor and anticancer drugs (Cryns and Yuan, 1998; Hengartner, 2000).

Trimerization of Fas receptor induced by Fas ligand recruits multiple procaspase-8 forming the death-inducing signaling complex (DISC) via the adapter molecule Fas-associated death domain (FADD) protein. FADD contains death domain (DD) and death effector domain (DED), through which FADD binds to Fas and procaspase-8, respectively, thereby linking Fas and procaspase-8 (Nagata, 1997). Procaspase-8, upon forming DISC, is activated autoproteolytically (Muzio *et al.*, 1998). The activated caspase-8 directly cleaves procaspase-3, an executioner caspase (Stennicke *et al.*, 1998). In the meanwhile, DNA-damaging agents such as anticancer drugs or irradiation induces apoptosis initiated at the mitochondrion by the release of cytochrome c, the mechanisms of which is not clarified yet (Sun *et al.*, 1999; Tepper *et al.*, 1999). The cytochrome c in the cytosol binds to apoptotic protease activating factor (Apaf)-1, and the binding recruits and activates procaspase-9 (Stennicke *et al.*, 1999). The activated caspase-9, in turn, also activates caspase-3 (Slee *et al.*, 1999; Hengartner, 2000). In some cells, caspase-8 activated by Fas truncates Bid, which translocates to mitochondria and stimulates the release of cytochrome c there. Thus, death signal generated by Fas can be transmitted to mitochondria, making up an amplification loop between mitochondria and caspases (Scaffidi *et al.*, 1998).

3-Deazaadenosine (DZA) which was developed as a potent inhibitor of S-adenosylhomocysteine (Ado-Hcy) hydrolase (Chiang *et al.*, 1977) serves as both an inhibitor and a substrate of this enzyme resulting in the accumulation of S-Ado-Hcy and 3-deazaadenosylhomocysteine (DZA-Hcy) (Chiang *et al.*, 1977; Aksamit *et al.*, 1982). DZA inhibits monocyte and neutrophil chemotaxis (Aksamit *et al.*, 1983), lymphocyte-mediated tumor cell lysis (Zimmerman *et al.*, 1978), macrophage phagocytosis and microfilament disorganization (Medzihradsky, 1984), histamine release by basophils (Morita *et al.*, 1982), macrophage lysosomal secretion (Riches *et al.*, 1985), and superoxide anion generation (Yagawa *et al.*, 1986). In addition, it has anti-human immunodeficiency

virus (HIV) activity (Flexner *et al.*, 1992), anti-inflammatory effect (Krenitsky *et al.*, 1986), and inhibitory effects of cytokine expression including tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1) (Jeong *et al.*, 1996), and nuclear factor- κ B (NF- κ B) transcription activity (Jeong *et al.*, 1999).

Recently, it has been demonstrated that DZA induced apoptosis in murine and human leukemic cells dependent on the activation of caspase-3-like activities, although the upstream events resulting in the activation of caspase-3-like activities remains to be clarified (Kim *et al.*, 1997; Kim *et al.*, 2000). In this study, we examined the activation pathway of the caspase cascade in the DZA-induced apoptosis.

Materials and Methods

Materials and reagents

3-Deazaadenosine (DZA) is a kind gift of Dr. Chiang of the Walter Reed Army Institute of Research, Washington DC. Caspase inhibitors were purchased from Bio-Rad Laboratories (Hercules, CA). Protease inhibitor cocktail was from Roche Molecular Biochemicals (Mannheim, Germany). Bongkreic acid (BA) and cyclosporin A (CsA) were from Calbiochem-Novabiochem (San Diego, CA). Agonistic anti-Fas antibody (CH11) and antagonistic anti-Fas antibody (ZB4) were from Upstate Biotechnology (Lexington, KY). 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was from Sigma (St. Louis, MO). All other reagents were of ACS or molecular biology grade, and from Sigma, otherwise specified.

Cell culture and viability assay

Human monocytic macrophage U-937 cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories Inc., Logan, UT) and 100 units/ml of penicillin/streptomycin in a humidified atmosphere of 95% air and 5% CO₂. To observe the effect of DZA on the growth of U-937 cells, cell viability was determined by MTT assay (Mossmann, 1983). Cells were seeded at a density of 1×10^4 /well in 96-well culture plates and then treated with indicated concentrations of DZA, finally adjusting media volume to 100 μ l. After incubation of indicated times, 10 μ l of MTT reagent (final concentration; 500 μ g/ml) was added to each well. Three hours later, cells were harvested and centrifuged to pellet MTT crystals. MTT crystals were dissolved with isopropanol containing 0.04 N of HCl and the absorbance was measured at 570 nm.

Evaluation of apoptosis

Induction of apoptosis was determined by fragmentation of genomic DNA through agarose gel electrophoresis

and fluorescence microscopic examination of cells stained with Hoechst 33342. Genomic DNA of cells treated with DZA or DZA plus other compounds for desired times was extracted using DNA extraction buffer (5 mM Tris-Cl, 20 mM EDTA, pH 8.0, 1% sodium dodecyl sulfate (SDS), 50 $\mu\text{g}/\text{ml}$ proteinase K) and extraction with phenol-ethanol. DNA pellet was dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 20 $\mu\text{g}/\text{ml}$ of RNase A, and incubated at 37°C for 1 h. Five micrograms of DNA was separated on 2% agarose gels with 0.5 x TAE (20 mM Tris-acetate, 1 mM EDTA). Ethidium bromide (EtBr)-stained DNA in the gel was visualized under UV light and photographed. To investigate morphological changes of nuclei in U-937 cells, cells were incubated in the presence of Hoechst 33342 (10 μM) for 10 min at 37°C. After being washed with phosphate-buffered saline (PBS), cells were examined under fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Antibodies

Mouse anti-caspase-3 antibody and mouse anti-caspase-9 antibody were purchased from Transduction laboratories (Lexington, KY). Rabbit anti-caspase-8 antibody was from Upstate Biotechnology (Lake Placid, NY). Mouse anti-cytochrome c antibody and mouse anti-porin antibody were from Pharmingen (San Diego, CA). Horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse IgG were from Sigma.

Western blot analysis

Cells treated as indicated were lysed with RIPA buffer (1% Triton X-100, 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA) containing protease inhibitor cocktail. Protein concentrations were measured by bicinchoninic acid method. Fifty micrograms of protein were resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto nitrocellulose membranes (Schleicher and Shuell, Dassel, Germany) in transfer buffer (25 mM Tris base, 193 mM glycine, 20% methanol). The membrane was soaked in 5% nonfat dried milk in tris-buffered saline (TBS; 10 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 30 min and then incubated with primary antibodies in blocking solution at 4°C overnight, followed by extensive washing with TTBS (TBS containing 0.05% Tween-20). Membranes were then incubated with peroxidase-conjugated secondary antibodies (goat anti-mouse or -rabbit IgG antibody) in blocking solution at room temperature, followed by washing with TTBS. Proteins were detected using the enhanced chemiluminescence Western blotting detection system (Amersham-Pharmacia, Buckinghamshire, UK).

Subcellular fractionation

Subcellular fractionation was performed according to the method of Akao *et al.* (1994). Briefly, harvested cells were washed twice with ice-cold PBS and then resuspended in hypotonic buffer (10 mM HEPES, 10 mM MgCl_2 , 42 mM KCl). Cells were passed through a 30-gauge syringe and centrifuged at 1,000 rpm for 5 min to remove unlysed cells and nuclei. The supernatant was further centrifuged at 15,000 rpm for 15 min at 4°C. The resulted pellet is heavy membrane fraction used as the mitochondrial fraction. The supernatant was ultracentrifuged at 100,000 g for 90 min. The pellet is light membrane fraction and the supernatant is cytosolic fraction.

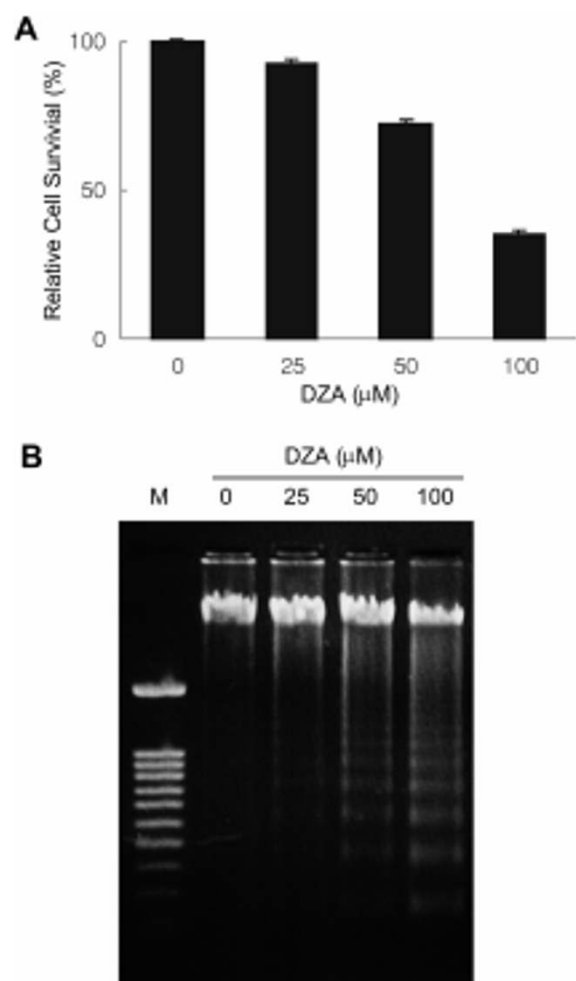


Figure 1. DZA-induced growth inhibition and DNA fragmentation in U-937 cells. (A) U-937 cells (1×10^4) were treated with the indicated concentrations of DZA. After 8 h, MTT assay was performed as described in Materials and Methods and expressed as relative cell survival which means the percent of cell viability of the treated cells to that of the cells without treatment. The data were expressed as mean \pm S.D. of three independent experiments performed in triplicate. (B) Genomic DNA of cells treated with indicated concentrations of DZA was extracted as described in Materials and Methods. Extracted genomic DNA were separated into 2% agarose gel. M: 100 bp DNA ladder marker.

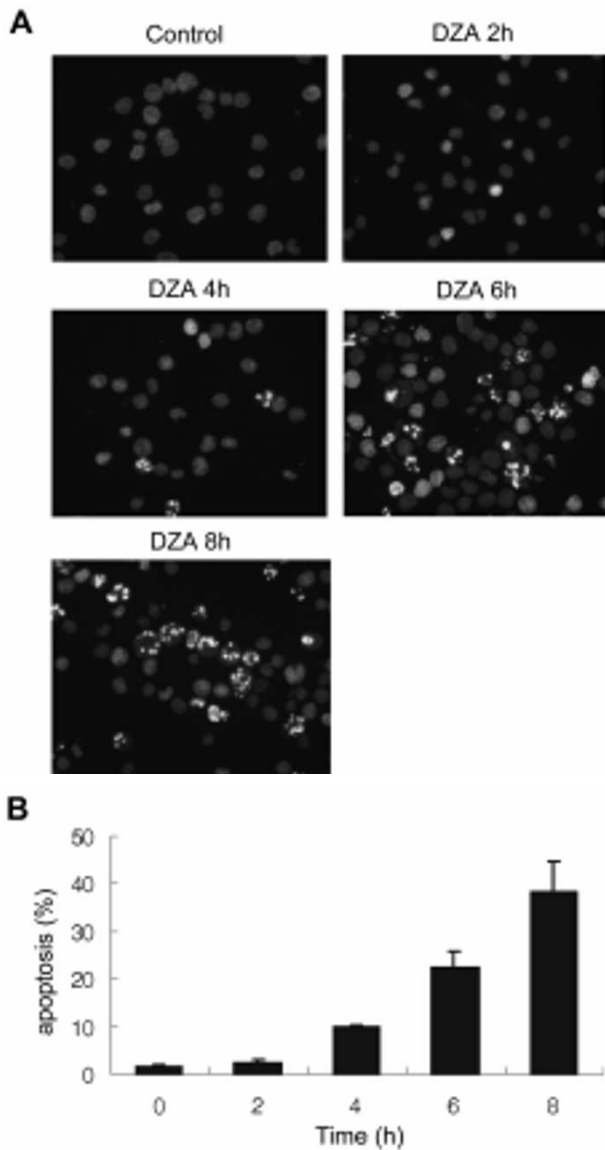


Figure 2. DZA-induced apoptosis in U-937 cells. (A) Cells treated with 100 μ M of DZA for indicated times were stained with Hoechst 33342 (10 μ M) for 10 min and observed under fluorescence microscope. (B) The number of the apoptotic cells were counted under fluorescence microscope. The data were expressed as mean \pm S.D. of three independent experiments performed in triplicate.

Results

Induction of U-937 cell apoptosis by DZA

DZA treatment of U-937 cells resulted in cell growth inhibition and fragmentation of nuclear DNA in a dose-dependent manner suggesting that the growth inhibitory effect of DZA is attributable to apoptosis (Figure 1). Fluorescence microscopic examination of Hoechst 33342-stained DZA-treated cells showed condensed and fragmented nuclei, characteristic features of apoptosis

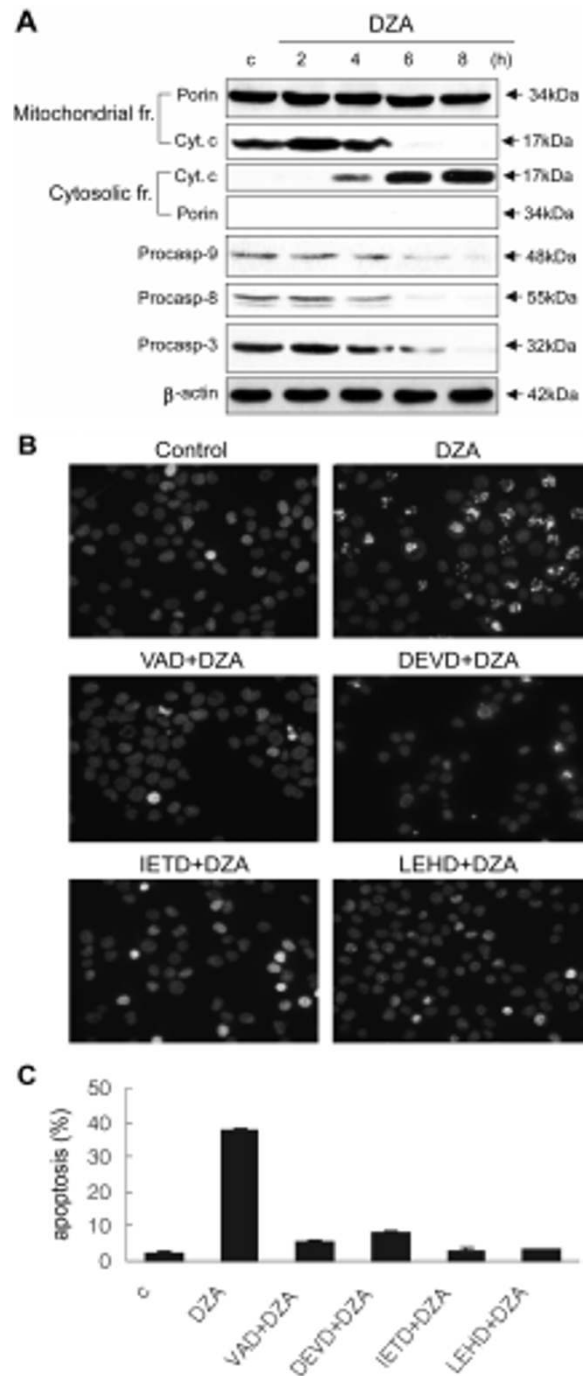


Figure 3. Activation of caspases cascade induced by DZA. (A) Subcellular fractions (fr) or total cell lysates of U-937 cells treated with 100 μ M of DZA were subjected to 15% or 10% SDS-PAGE respectively, and then Western blot analysis was performed with anti-cytochrome c antibody and anti-caspases-9, -8, -3 antibodies as described in Material and Methods. (B), (C) Cells were incubated in the absence or presence of caspase inhibitors (100 μ M of zVAD-Fmk, 200 μ M of zDEVD-Fmk, 200 μ M of zIETD-Fmk, 400 μ M of zLEHD-Fmk) for 1 h and treated for 8 h further with 100 μ M of DZA. Cells were stained with Hoechst 33342 (10 μ M) and examined under fluorescence microscope (B) and the number of apoptotic cells were counted (C). The data were expressed as mean \pm S.D. of three independent experiments performed in triplicate.

(Figure 2a). The level of apoptosis was highest in U-937 cells treated with 100 μ M of DZA for 8 h (Figure 2b). Thus, 100 μ M concentration of DZA was used in all subsequent experiments.

Release of cytochrome c and activation of caspase cascade induced by DZA

Cytochrome c release from mitochondria and the activation of caspase-9, -8 and -3 in the U-937 cells treated with DZA was examined by Western blot analysis. As shown in Figure 3, the cytochrome c was released to cytosol post 4h DZA treatment and activation of caspase-9, -8 and -3 was also observed. Porin was used as a reference mitochondrial protein for subcellular fractionation. The early cellular apoptotic responses associated with cytochrome C release post 4 h DZA-treatment suggest that possible relationship between cytochrome c release and caspase cascade in U-937 cells affected by DZA.

To examine the involvement of caspases in this system, the effect of specific caspase inhibitors was tested on apoptosis of the U-937 cells in the presence or absence of DZA. Pretreatment of cells with caspase inhibitors: a universal caspase inhibitor zVAD-Fmk, a caspase-3 inhibitor zDEVD-Fmk, a caspase-8 inhibitor zIETD-Fmk, and a caspase-9 inhibitor zLEHD-Fmk, blocked nuclear fragmentation and condensation induced by DZA (Figure 3B and C). Thus, the activation of caspase-9, -8 and -3 seems to be necessary to this apoptosis.

Effect of caspase inhibitors on DZA-induced activation of caspase-9, -8 and -3

To determine possible steps involved in DZA-induced caspase activation associated with cellular apoptosis, the effect of specific individual caspase inhibitor on the activation of caspase-9, -8, and -3 was examined by Western blot analysis (Figure 4). zDEVD-Fmk and zLEHD-Fmk prevented the activation of caspase-8, but not the activation of caspase-3 and caspase-9 indicating that caspase-8 is downstream of caspase-3 and caspase-9 which are activated by independent pathways. The activation of caspase-3 was inhibited weakly by zIETD-Fmk but almost completely by zVAD-Fmk, suggesting that other caspase activities including IETDase activity seem to be present upstream of caspase-3. Being consistent with other reports that caspase-9 is activated directly by cytochrome c (Stennicke *et al.*, 1998), caspase inhibitors used exerted no effect on the activation of caspase-9 ruling out the involvement of caspases on the activation of itself.

The effect of antagonistic Fas-Ab (ZB4) on DZA-induced caspase-8 activation

Chemotherapeutic agents such as doxorubicin is reported to activate caspase-8 by inducing Fas receptor-ligand interaction, preceding the activation of caspase-3 (Friesen *et al.*, 1996; Fulda *et al.*, 2000). To study whether the Fas receptor-ligand interaction is involved in the DZA induced caspase-8 activation, U-937 cells

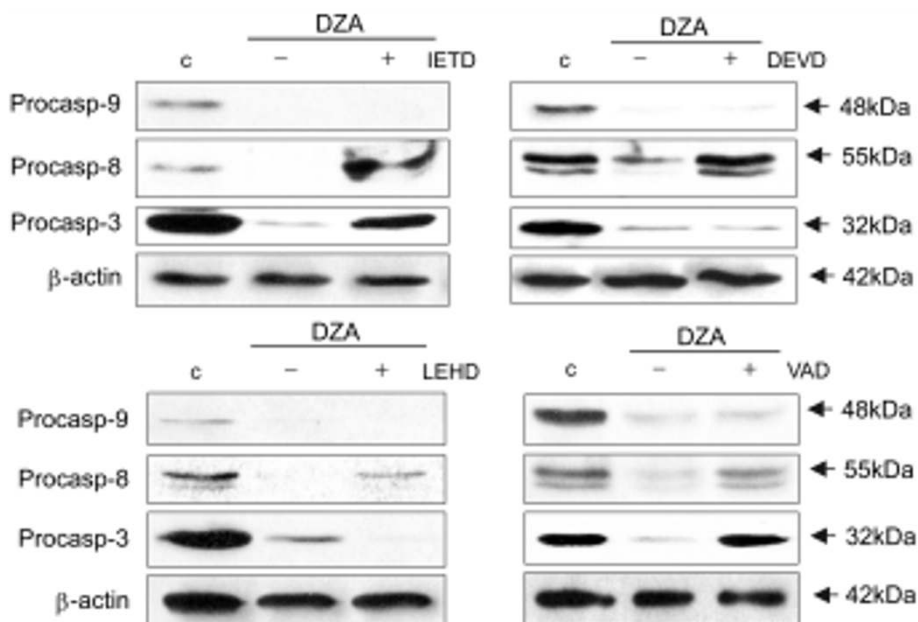


Figure 4. The effect of specific caspase inhibitors on the activation of caspase -9, -8, -3 induced by DZA. Cells were pretreated for 1 h with caspase inhibitors (100 μ M of zVAD-Fmk, 200 μ M of zDEVD-Fmk, 200 μ M of zIETD-Fmk, 400 μ M of zLEHD-Fmk) and further incubated for 8 h in the presence of DZA (100 μ M). Cell lysates were subjected to 10% SDS-PAGE and then Western blot analysis was performed with anti-caspases-9, -8, and -3 antibodies.

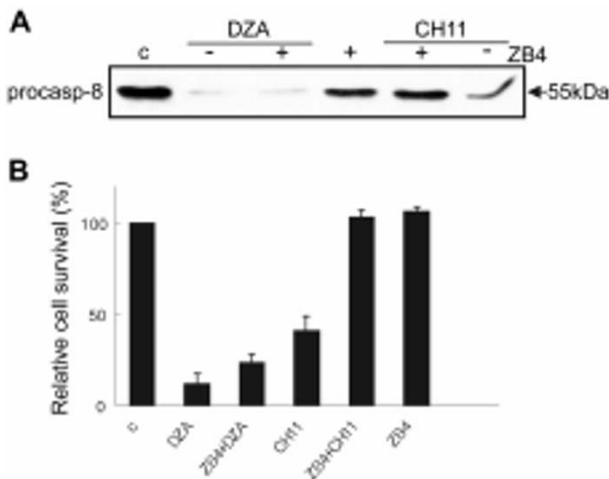


Figure 5. The effect of antagonistic Fas-Ab (ZB4) on the activation of caspase-8 induced by DZA. (A) Cells pretreated for 1 h with 500 ng/ml of ZB4 or media alone were incubated for 8 h with DZA (100 μ M) or CH11 (agonistic Fas-Ab; 1 μ g/ml). Cell lysates were subjected to 10% SDS-PAGE and then Western blot analysis was performed with anti-caspase-8 antibody. (B) Cells (1×10^4) were treated as the Figure 5(A) legend. Cell viability was determined by MTT assay and expressed as relative cell survival which means the percent of cell viability of the treated cells to that of the cells without treatment. The data were expressed as mean \pm S.D. of three independent experiments performed in triplicate.

were treated with the antagonistic Fas-Ab (ZB4) before the addition of DZA. ZB4 inhibited the caspase-8 activation and reduction of cell viability induced by agonistic Fas-Ab (CH11), but it exerted no effect on the DZA induced cellular events (Figure 5). Thus, the Fas receptor-ligand system does not appear to be involved in the activation of caspase-8 and the apoptosis induced by DZA, confirming that the caspase-8 activation is downstream of caspase-3.

Effect of bongkreik acid (BA), cyclosporin A (CsA), and zVAD-Fmk on DZA-induced cytochrome c release

CsA and BA, known inhibitors of cytochrome c release by closing the mitochondria permeability transition pore (MPTP) (Crompton, 1999; Jacotot *et al.*, 2000) induce prevention of apoptosis in several systems. The effects of BA, CsA and zVAD-Fmk on the release of cytochrome c induced by DZA was examined to understand the mechanism involved in the release of cytochrome c and its contribution to apoptosis. Neither BA nor CsA altered the translocation of cytochrome c from mitochondria to cytosol (Figure 6) and apoptosis (data not shown). zVAD-Fmk which induced prevention of apoptosis did not affect the release of cytochrome c. These results suggest that DZA stimulate the release of cytochrome c through other pathways than CsA- or BA-inhibitory MPTP, and cytochrome c release is upstream and independent of caspase activation.

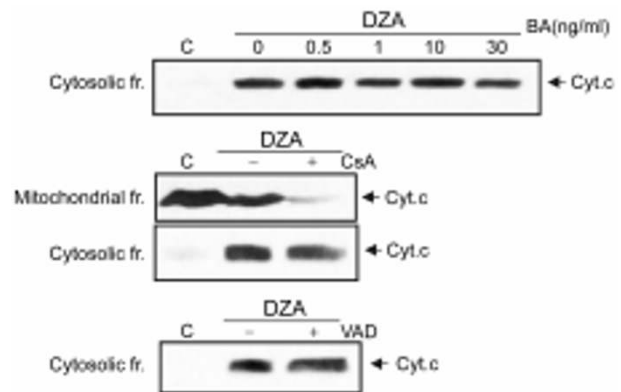


Figure 6. The effect of BA, CsA, and zVAD-Fmk on the release of cytochrome c induced by DZA. Cells pretreated for 1 h with indicated concentrations of BA, 20 μ M of CsA or 200 μ M of zVAD-Fmk were incubated for 8 h with 100 μ M of DZA. Aliquots of subcellular fractions or total cell lysates were separated by 15% SDS-PAGE and subjected to Western blot analysis.

Discussion

DZA-induced apoptosis of human leukemic cells was caused by activating caspase-3-like activities (Kim *et al.*, 2000). In this study, the activation pathway of caspase cascade in the DZA-induced apoptosis was investigated by analyzing the effects of specific caspase inhibitors on the activation of caspase-9, -8 and -3. DZA stimulated the release of cytochrome c, activation of caspase-9, -8 and -3 before inducing apoptosis. zDEVD-Fmk, a caspase-3 inhibitor, and zLEHD-Fmk, a caspase-9 inhibitor, blocked the activation of caspase-8 but neither caspase-3 nor caspase-9, suggesting that caspase-8 is activated by caspase-9 and caspase-3 which are activated independently. The activation of caspase-3 was completely abolished by a universal inhibitor of caspases zVAD-Fmk, and slightly by a caspase-8 inhibitor zIETD-Fmk, indicating that caspase-3 is activated by other active caspases including caspase-8-like enzymes but not caspase-8, because at this concentration of zIETD-Fmk, the activation of caspase-8 was completely prevented. Caspase inhibitors did not alter the activity of caspase-9 and the release of cytochrome c. So, it appears that DZA-induced cytochrome c release is independent of caspase activities except caspase-9 that is activated directly by released cytochrome c. These data can be summarized schematically as shown in Figure 7. DZA activates two pathways; one is caspase-independent cytochrome c release and subsequent activation of caspase-9, and the other is caspase-dependent activation of caspase-3. The activated caspase-3 and caspase-9, in turn, activates caspase-8. Because zLEHD-Fmk and zDEVD-Fmk, which inhibited the activation of caspase-8 but neither caspase-3 nor caspase-9, and zIETD-Fmk prevented apoptosis, caspase-8

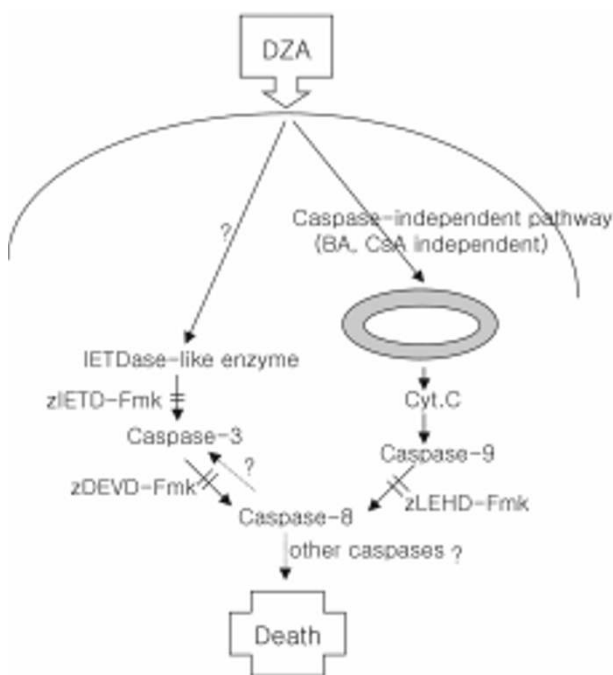


Figure 7. The schematic diagram of caspase cascade activated by DZA.

seems to act as an executioner caspase in the DZA-induced apoptosis.

In general, caspase-8 acts as an upstream caspase relaying death signal generated by Fas receptor-ligand interaction to downstream caspase-3, directly cleaving procaspase-3 or to mitochondria *via* truncated Bid (Kruidering and Evan, 2000). In addition, it has been shown that caspase-8 can process many other caspases (Srinivasula *et al.*, 1996), implying that caspase-8 may act as an initiator caspase. However, in the apoptosis induced by anticancer drugs, the role and location of caspase-8 is controversial. Doxorubicin, cisplatin and VP-16 induced apoptosis in human leukemic and neuroblastoma cells by activating Fas receptor-ligand interaction suggesting the role of caspase-8 as an initiator caspase (Friesen *et al.*, 1996; Fulda *et al.*, 1997). But, Fas receptor-ligand interaction was not involved in the apoptosis and the activation of caspase-8 of leukemic cells such as HL-60, K562, and U-937 cells induced by anticancer drugs, although the role of caspase-8 was not definitely determined (McGahon *et al.*, 1998; Tolomeo *et al.*, 1998). Recently, caspase-8 as an executioner caspase in the anticancer drug-induced apoptosis was reported (Engels *et al.*, 2000). The discrepancies about the role of caspase-8 may be related to cell type differences rather than anticancer agent itself, since doxorubicin induces Fas-dependent (Friesen *et al.*, 1996) and -independent apoptosis (McGahon *et al.*, 1998). In cells with high levels of caspase-8 and inducible expression of Fas and Fas ligand, anticancer drugs might induce apoptosis by way of Fas receptor-ligand

system. In other cell lines with low levels of caspase-8 and are incapable of inducing Fas and Fas ligand, Fas receptor-ligand system seems not to be involved in the apoptosis by anticancer drugs. U-937 cell line used in this experiment expresses Fas and Fas ligand, the levels of which were not changed by DZA (data not shown) being consistent with a previous report (McGahon *et al.*, 1998). So, the apoptosis and the activation of caspase-8 in U-937 cells might be mediated by Fas-independent pathway. As expected, antagonistic Fas-Ab (ZB4) exerted no effects on the apoptosis and the activation of caspase-8 in U-937 cells induced by DZA. Because it was not examined the effect of DZA on other cell lines having Fas-dependent apoptotic mechanism, we don't know yet whether the apoptotic mechanism in U-937 cells induced by DZA is cell-type specific.

In these experiments, in contrast to the fact that caspase-8 is an upstream caspase and can activate caspase-3, by directly cleaving procaspase-3, and caspase-9 by stimulating the release of cytochrome c from mitochondria, caspase-8 was activated downstream of caspase-3 and caspase-9. Although the mechanism involved in the activation of caspase-8 by caspase-3 or -9 is not clarified in this study, this finding is consistent with other reports that caspase-8 is a downstream effector of caspase-3 or -9 (Slee *et al.*, 1999; Wieder *et al.*, 2001). Since zDEVD-Fmk and zLEHD-Fmk, inhibitors of caspase-3 and caspase-9, respectively, blocked the processing of procaspase-8 and apoptosis, caspase-8 needs both caspase-3 and caspase-9 to be activated.

It has been suggested that permeability transition, and a subsequent drop in mitochondrial membrane potential, is a principal mechanism by which cytochrome c is released from the mitochondria into the cytosol during apoptosis (Bernardi *et al.*, 1999). Thus, inhibitors of mitochondrial permeability transition (MPT) such as BA and CsA, are able to inhibit apoptosis induced by cytochrome c-involved caspase cascade (Crompton, 1999; Jacotot *et al.*, 2000). The adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), Bax, and cyclophilin D are known to constitute the MPTP at the mitochondrial inner and outer membrane contact region (Beutner *et al.*, 1998; Woodfield *et al.*, 1998). BA, a ligand of ANT and CsA, a ligand of cyclophilin D, inhibit pore formation by the ANT, thereby closing the MPTP (Kroemer and Reed, 2000).

In these experiments, the release of cytochrome c in U-937 cells was stimulated by DZA and was not affected by BA and CsA. Therefore, it seems that MPTP and permeability transition may not involved in the release of cytochrome c by DZA like didemnin B (Grubb *et al.*, 2001). But, because we did not directly measure the mitochondrial membrane potential, it cannot be definitely concluded whether the involvement of permeability transition. Recently, Genini *et al.* (2000) reported that deoxyadenosine analogs such as 2-deoxy-

adenosine, 2-chloro-2-deoxyadenosine and 2-chloro-2-fluorodeoxyadenosine directly disrupted the integrity of mitochondria, suggesting the binding of deoxyadenosine analogs and the ANT, before inducing apoptosis in primary chronic lymphocytic leukemic cells. Therefore, it can be postulated, then, that DZA may bind to the ANT or other mitochondrial proteins not affected by BA or CsA and stimulate the release of cytochrome c, although more intensive experiments should be performed to understand molecular mechanisms involved in the release of cytochrome c.

zVAD-Fmk, which completely blocked the apoptosis and the activation of caspase-8, had no effect on the release of cytochrome c, ruling out the involvement of caspases including caspase-8 and truncated Bid on mitochondrial events. This finding confirms that cytochrome c release is upstream and independent of caspase activation.

In summary, DZA induced apoptosis in U-937 cells by activating a peculiar caspase cascade in that both caspase-dependent and -independent cascades were activated simultaneously, and caspase-8 was activated downstream of caspase-3 and caspase-9, serving as an executioner caspase. But, more definitive molecular experiments should be performed to investigate the exact mechanisms involved in the DZA-induced apoptosis. These studies will provide us good information about the caspase cascade and increase the therapeutic effectiveness of DZA and other anti-cancer drugs.

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