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Up-regulation of Bax and endonuclease G, and down-modulation of Bcl-X_L involved in cardiotoxin III-induced apoptosis in K562 cells

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Abbreviations: CTX III, Carditoxin III; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Abstract

Cardiotoxin III (CTX III), a basic polypeptide with 60 amino acid residues isolated from *Naja naja atra* venom, has been reported to have anticancer activity. CTX III-induced K562 cell apoptosis was confirmed by DNA fragmentation (DNA ladder, sub-G1 formation) and phosphatidylserine (PS) externalization with an IC_{50} value of 1.7 μg/ml at 48 h. A mechanistic analysis demonstrated that CTX III-induced apoptotic cell death was accompanied by up-regulation of both Bax and endonuclease G (Endo G), and downregulation of Bcl-X_L. CTX III had no effect on the levels of Bcl-2, Bid, XIAP survivin, and AIF proteins. CTX III treatment caused loss of the mitochondrial membrane potential (△Ψm), release of mitochondrial cytochrome c to the cytosol, and activation of both caspase-9 and -3. CTX III-induced apoptosis was significantly blocked by the broad-spectrum caspase inhibitor Z-VAD-FMK. However, CTX III did not generate reactive oxygen species (ROS) and antioxidants, including N-acetylcysteine and catalase, did not block CTX III-induced apoptosis in K562 cells. Modulation of Bax, Bcl-X_L, and the Endo G proteins, release of mitochondrial cytochome c, and activation of caspase-3 and -9 all are involved in the CTX III-triggered apoptotic process in human leukemia K562 cells.

Keywords: apoptosis; bcl-2-associated X protein; cardiotoxin III, Naja naja atra; caspase; endonuclease G; K562 cells

Introduction

Many therapeutic and chemopreventive agents eliminate cancerous cells by inducing programmed cell death (apoptosis) (Kaufmann *et al.*, 2000; Robertson *et al.*, 2000). Apoptosis is an important cellular process for destruction of undesirable cells during development or homeostasis in multi-cellular organisms. This process is characterized by distinct morphological changes including plasma membrane bleb, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (Kaufmann *et al.*, 2001; Reed, 2001). Caspases are essential for the execution of cell death by various apoptotic stimuli (Cohen, 1997; Shi, 2002). Caspase activation is often regulated by various cellular proteins including members of the IAP (Deveraux and Reed, 1999) and Bcl-2 families (Adams and Cory, 1998; Antonsson and Martinou, 2000). Previous reports have demonstrated that some Bcl-2 family members that are located on the mitochondrial membrane can alter the permeability of the mitochondrial membrane and trigger the release of cytochrome c (Adams and Cory, 1998; Antonsson and Martinou, 2000) or caspases (Salvesen and Dixit, 1997), then activate the post-mitochondrial caspase cascade leading to apoptotic cell death. Apoptosis induced by anticancer or chemopreventive agents can be mediated by additional proteins or pathways including release of apoptogenic factors, such as endonuclease G (endo G) and apoptosis-inducing factor (AIF), from the mitochondrial intermembrane space into the cytosol (Green and Reed, 1998; Ravagnan *et al.*, 2002), or by oxidative stress, such as reactive oxygen species (ROS) (Fleury *et al.*, 2000; Simon *et al.*, 2000).

The aim of this study was to identify the mechanisms of CTX III-induced apoptosis. We show that CTX III-induced apoptosis in human leukemia cancer cells is caspase-3 and -9 dependent, is blocked by a pan-caspase inhibitor, and is accompanied by up-regulation of Bax and Endo G proteins, down-regulation of Bcl-X_L, and an increase in the release of mitochondrial cytochrome c to cytosol.

Materials and Methods

Materials

RPMI 1640 medium, fetal calf serum (FCS), trypan blue, penicillin G, and streptomycin were obtained from GIBCO BRL (Gaithersburg, MD). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), ribonuclease (RNase), propidium iodide (PI), rhodamine123, N-acetyl-L-cysteine (NAC), and catalase were purchased from Sigma-Aldrich (St. Louis, MO) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Molecular Probes, Inc (Eugene, OR). Antibodies were obtained from the following sources: cytochrome c (PharMingen, San Diego, CA), Bax, Bcl-2, Bcl-X_L, Bid, XIAP, AIF, Survivin, Endo G, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Hybond ehanced chemiluminescence (ECL) transfer membrane and ECL Western blotting detection kit were obtained from Amersham Life Science (Buckinghamshire, UK). The colorigenic synthetic peptide substrate, Ac-DEVD-pNA, and Ac-LEHD-pNA, as well as the protease inhibitor for Z-VAD-FMK were purchased from Calbiochem (San Diego, CA). An annexin V-FLOUS Staining Kit was a product of Roche Molecular Biochemicals (Mannheim, Germany).

CTX III was purified from the venom of *Naja naja atra* (Taiwan cobra) by chromatogaraphy on Sephadex G-50 and SP-Sephadex C-25, as previously described by Lin *et al.,* (2002). Solutions of CTX III were prepared in phosphate buffered saline (PBS) and sterilized by filtration.

Cell culture

Human leukemia K562 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin) at 37°C in a humidified atmosphere of 5% CO_2.

Cell viability and cytotoxicity

Cell viability was determined by the Trypan blue dye exclusion method and cytotoxicity was assessed by an MTT assay. Exponentially growing cells (1×10^5) were plated in 96-well plates and, after 24 h of growth, were treated with a series of different concentrations of CTX III dissolved in PBS. Cells exposed to 0.2% Trypan Blue were counted in a hemocytometer. MTT solution was added to each well (1.2 mg/ml) and incubated for 4 h. This reaction results in mitochondrial dehydrogenases of viable cells producing a purple formazan product. The amount of MTT-formazan product dissolved in DMSO was estimated by measuring the absorbance at 570 nm in an ELISA plate reader.

Assessment of apoptosis

Apoptosis was also evaluated by examining the characteristic pattern of DNA laddering generated in apoptotic cells using gel electrophoresis. Briefly, cells were seeded at a density of 1×10^6 cells onto 10-cm dishes 24 h before CTX III treatment. Then cells were treated with various concentrations of CTX III for 12 h and control cultures were treated with PBS. Adherent and floating cells were collected and lysed in 400 μl of ice-cold lysis buffer (containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.3% Triton X-100), incubated on ice for 30 min, and then centrifuged. RNAase (100 μg/ml) was added to the supernatant, which was then incubated at 50°C for 30 min, followed by addition of 200 μg/ml of proteinase K and further incubation at 37°C for 1 h. Fragmented DNA was extracted with phenol /chloroform and then precipitated with ethanol. The DNA fragments were electrophoresed on a 2% agarose gel containing 0.1 μg/ml of ethidium bromide.

The accumulation of the sub G1 population in K562 cells was also determined by flow cytometry. Cells were seeded onto 6 cm dishes and treated with or without the indicated CTX III for 24 h. Cells were then washed twice with ice-cold PBS and collected by centrifugation at 200 *g* for 5 min at 4°C. Cells were fixed in 70% (v/v) ethanol at 4°C for 30 min. After fixation, cells were treated with 0.2 ml of DNA extraction buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid buffer, pH 7.8) for 30 min, centrifuged, and resuspended in 1 ml of propidium iodide staining buffer (0.1% TritonX-100, 100 μg/ml RNase A, 500 μg/ml of propidium iodide in PBS) at 37°C for 30 min. Cytometric analyses were performed using a flow cytometer (FACS Calibur, Becton Dickinson) and CellQuest software. Approximately 10,000 cells were counted for each determination.

The externalization of phosphatidylserine (PS) and membrane integrity were quantified using an Annexin V-FLOUS staining kit. In brief, 10^6 cells were grown in 35 mm diameter plates and were labeled with Annexin V-FLOUS (10 μg/ml) and PI (20 μg/ml) prior to harvesting. After labeling, all plates were washed with binding buffer and harvested by scraping. Cells were resuspended in binding buffer at a concentration of 2×10^5 cells/ml before analysis by flow cytometry.

Western blot analysis

Cells were treated with CTX III for the indicated time periods. After incubation, cells were lysed in modified protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5% 2-mercaptoethanol, 1% Nonidet P-40, 0.25% Na deoxycholate, 5 g/ml of leupeptin, 5 μg/ml of aprotinin, 10 μg/ml of soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonylfluoride), then the protein concentration of the supernatant was measured using the BCA reagents (Pierce, Rockfold, IL). Equal amounts of sample lysate were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto PVDF membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), and incubated overnight at 4°C with specific primary antibodies. The membrane was subsequently washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies. Determinations were made using ECL kits (Amersham, ECL Kits).

Preparation of the cytosolic fraction for assessment of cytochrome c, AIF, and Endo G

The mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml of phenylmethylsulfonylfluoride (PMSF), 8 μg/ml of aprotinin, and 2 μg/ml of leupeptin, pH 7.4). Cells were passed through a needle 10 times. Unlysed cells and nuclei were pelleted by centrifugation for 10 min at 750 × *g*. The supernatant was then centrifuged at 100,000 × *g* for 15 min. This pellet, representing the mitochondrial fraction, was resuspended in buffer A. The supernatant was again centrifuged at 100,000 × *g* for 1 h. The supernatant from this final centrifugation step represented the cytosolic fraction.

Assays of caspase-3 and-9 activities

After different treatments, cells (10^6 cells/ml) were collected and washed three times with PBS and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 18,000 × *g* for 3 min and clear lysates containing 50 μg of protein were incubated with 100 μM enzyme- specific colorigenic substrates at 37°C for 1 h. The alternative activities of caspase-3 and -9 were determined as cleavage of the colorimetric substrate by measuring the absorbance at 405 nm.

Flow cytometric detection of ROS and $\triangle\Psi m$

To evaluate the intracellular ROS level, CTX III-treated cells were incubated with 5 μM H_2DCF-DA for 30 min at 37°C. After treatment with H_2DCF-DA, the cells were washed twice with PBS, then resuspended in PBS and analyzed on a flow cytometer (FACScan, Becton Dickinson, San Jose, CA.) equipped with a single 488 nm argon laser. The 2',7'-dichlorofluorescein (DCF) data were recorded using an FL1 photomultiplier. To assess the change in $\triangle\Psi m$, CTX III-treated cells were incubated with 40 nM rhodamine 123 for 15 min at 37°C. The rhodamine 123 data were recorded using an FL1

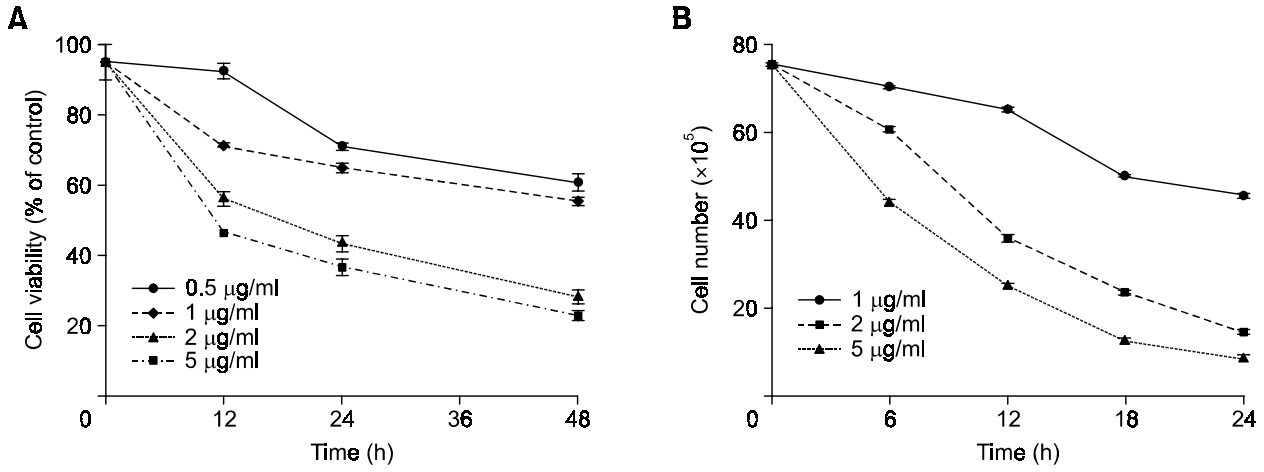

Figure 1. Effect of CTX III on proliferation of K562 cells determined by (A) MTT and (B) trypan blue dye exclusion assay. Cells were incubated with a series of different concentrations of CTX III for 48 h, and cell survival was then determined. The percentage of viable cells was calculated as a ratio of treated to control cells (treated with PBS). Data are presented as mean ± SD of three independent experiments.

photomultiplier. Sample data (10,000 cells) were used to prepare histograms on the Cell Quest data analysis program (Becton Dickinson). Dead cells and debris were excluded from the analysis by electronic grating of forward and side scatter measurements.

Statistical analysis

All data were expressed as the mean ± SD. Differences between treated and control cells were analyzed by student's *t*-test. A probability of $P < 0.05$ was considered significant.

Results

CTX III Inhibits the proliferation of K562 cells through the induction of apoptosis

To verify the effect of CTX III on cell growth, K562

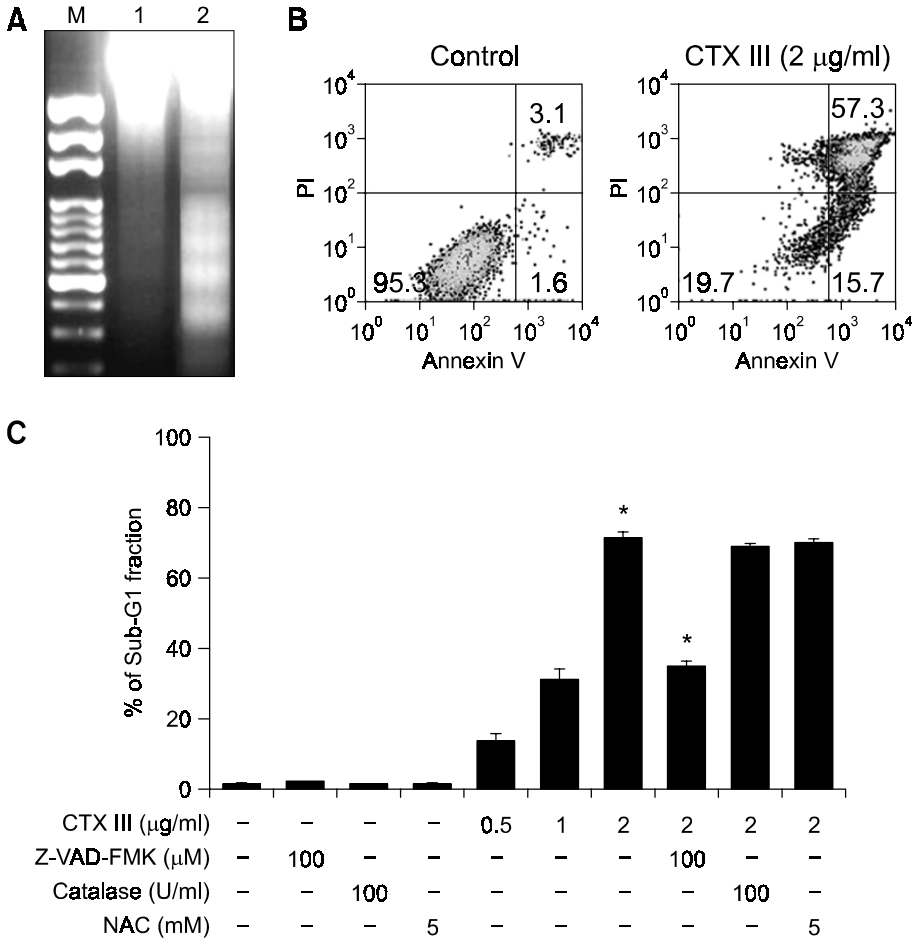

Figure 2. CTX III induces apoptosis in K562 cells. (A) Dose-dependent DNA fragmentation. Cells were exposed to the indicated concentrations of CTX III for 12 h. Cells were harvested by centrifugation and DNA was extracted. The DNA fragments were separated on 2% agarose gel electrophoresis and were visualized under ultraviolet light after staining with ethidium bromide. M: size marker (100 base pair DNA ladder); Lane 1, control culture; Lane 2, 2 μg/ml CTX III treatment (B) Flow cytometric analysis of phosphatidylserine externalization (annexin V binding) and cell membrane integrity (PI staining). Cells were treated with 2 μg/ml of CTX III for 12 h. The dual parameter dot plots combining annexin V-FITC and PI fluorescence show the vial cell population in the lower left quadrant (annexin V^-PI^-), the early apoptotic cells in the lower right quadrant (annexin V^+PI^-), and the late apoptotic cells in the upper right quadrant (annexin V^+PI^+). (C) The sub G1 DNA content in K562 cells by flow cytometry. Cells were pre-incubated with or without the caspase inhibitor Z-VAD-FMK, N-acetylcysteine (NAC), and catalase for 1h at the concentration indicated, followed by treatment with or without the indicated concentrations of CTX III for 24 h. After fixing in 70% ethanol, cells were stained by adding propidium iodide. The DNA content was analyzed using Cell Quest software with a flow cytometry system. Data are presented as mean and SD with $n = 3$. * $P < 0.05$.

cells were treated with increasing concentrations of CTX III for 48 h, and cell survival was assessed by an MTT assay. As shown in Figure 1A, a significant loss of viability was detected at 0.5, 1, 2, and 5 μg/ml of CTX III in a dose- and time-dependent manner with an IC_{50} value of 1.7 μg/ml at 48 h. Cell growth inhibition by CTX III was further confirmed using the trypan blue dye exclusion method, and the results are shown in Figure 1B. Proliferation of K562 cells was significantly suppressed in the presence of CTX III in a concentration-dependent manner. The dose of 1.7 μg/ml that was used to kill K562 cancer cells was lower than the 10 μg/ml that was required to enhance apoptosis of PHA-activated T cells, indicating that transformed cells are more susceptible to this toxin (Su *et al.*, 2003).

To determine whether CTX III decreases cell survival by the induction of apoptosis, we analyzed chromosomal DNA fragmentation by agarose gel electrophoresis. As shown in Figure 2A, CTX III induced apoptosis, as shown by the formation of distinct internucleosomal DNA fragmentation. To quantify the degree of apoptosis, we analyzed the amount of sub-G1 DNA by flow cytometry. As shown in Figure 2C, CTX III treatment in K562 cells resulted in a markedly increased accumulation of cells in the sub-G1 phase in a dose-dependent manner. Simultaneous staining with Annexin V-FLOUS and PI allowed differentiation of intact cells and early apoptosis vs. late apoptosis (Van Engeland *et al.*, 1998). In the K562 control culture, 95.3% of cells were viable, 1.6% were in early apoptosis, and 3.1% were in late apoptosis (Figure 2B). In K562 cells treated with CTX III (2 μg/ml) for 12 h, 15.7% of cells were in early apoptosis and 57.3% were in late apoptosis (Figure 2B). Evidently, the mechanism of cytotoxicity induced by CTX III in K562 cells is related to the apoptosis-inducing activity.

Effect of CTX III on the expression levels of the Bcl-2 and IAP family proteins

To explore the possible role of Bcl-2 family members in CTX III-induced apoptosis, we examined the effects of CTX III on the expression levels of Bcl-2 members by Western blot analysis. Exposure of K562 cells to 2 μg /ml of CTX III resulted in down-regulation of Bcl-X_L expression. In contrast, CTX III significantly upregulated the expression of Bax, while the expression levels of Bcl-2 and Bid proteins were unaltered (Figure 3 A).

In addition to Bcl-2 family proteins, the IAP family proteins regulate apoptotic signaling cascades by blocking caspase activities (Takahashi *et al.*, 1998). However, the expression levels of XIAP and survivin were not altered by treatment with CTX III (Figure 3B).

Release of mitochondrial nucleases

AIF and the recently discovered Endo G are two nucleases that are located in the mitochondrial intermembrane space (Green and Reed, 1998; Ravagnan *et al.*, 2002). Release of these nucleases into the cytosol results in DNA laddering, with AIF inducing large-scale fragmentation (> 50 kbp) and Endo G inducing nucleosome-size fragmentation (Li *et al.*, 2001). As shown in Figure 3C, Endo G was released from mitochondria after CTX III treatment, while AIF was not affected (Figure 3C).

Caspases mediate CTX III induced apoptosis

To determine whether activation of caspase-3 and -9 plays a role in CTX III-induced apoptosis, the activities of caspase-3 and -9 during CTX III-induced

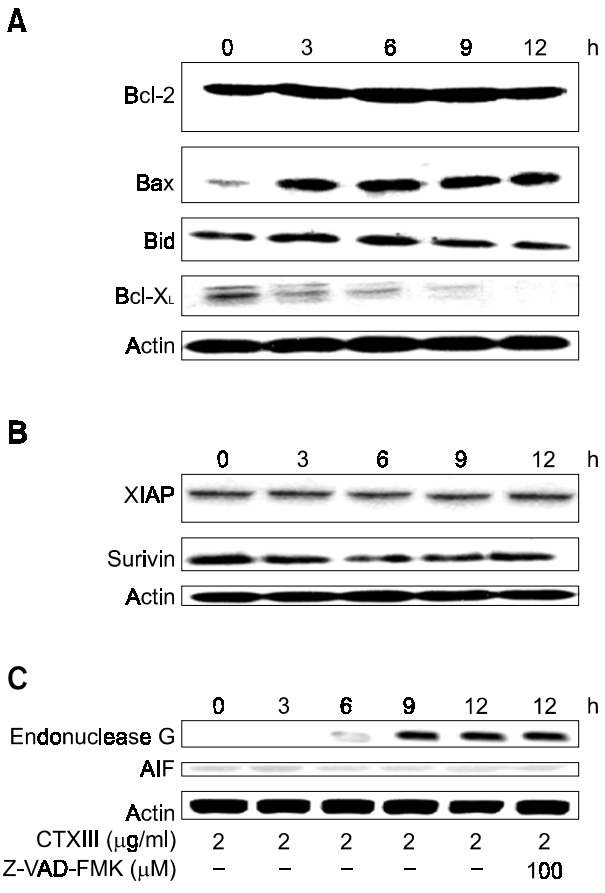

Figure 3. Western blot analysis of (A) modulation of Bcl-2 family proteins, (B) expressions of XIAP and survivin, and (C) expressions of the AIF and Endo G proteins. Cells were treated with CTX III (2 μg/ml) for the indicated time periods. After treatment, the fractions were resolved by SDS-PAGE, transferred onto cellulose membranes, then probed with specific antibodies and visualized using a chemiluminescence, ECL kit. The amount of β-actin was measured as an internal control. Each blot is representative of three independent experiments.

apoptosis were measured by a decrease in the proenzyme level measured by Western blot analysis and the proteolytic activity with a chromogenic substrate. As shown in Figure 4A, treatment with CTX III resulted in a decrease in the levels of Procaspase-3 and -9 in K562 cells exposed to 2 μg/ml of CTX III for 12 h. To verify and quantify the proteolytic activities of caspase-3 and -9, we performed an *in vitro* assay based on the proteolytic cleavage of chromophore p-nitroanilde (pNA) from specific colorigenic peptide substrates (Ac-DEVD-pNA for caspase-3 and Ac-LEHD-pNA for -9). A marked time-dependent increase in the activities of caspase-3 and -9 was observed in K562 cells treated with 2 μg/ml of CTX III (Figure 4B). To confirm that apoptosis due to CTX III occurs via the caspase dependent pathway, cells were pre-incubated with 100 μM of Z-VAD-FMK, a caspase inhibitor, for 1h before treatment with 2 μg/ml of CTXIII for 24 h. Z-VAD-FMK significantly reduced the population of apoptotic cells induced by CTX III in comparison with an untreated control (Figure 2C). These data clearly indicate that CTX III-induced apoptosis is associated with caspase-9 and caspase-3 activation.

CTX III-induced apoptosis involves mitochondrial cytochrome c release and loss of $\triangle\Psi m$

The release of cytochrome c from mitochondria into the cytosol is one of the major apoptosis pathways (Green and Reed, 1998; Jiang and Wang, 2000). To determine whether cytochrome c is released in CTX III-induced apoptosis, we prepared cytosolic fractions from CTX III-treated cells and detected cytochrome c by Western blotting analysis. As shown in Figure 4C, a time-dependent accumulation of cytochrome c in the cytosol was observed in CTX III-treated cells. Since cytochrome c release is linked to the loss of $\triangle\Psi m$ (Henry-Mowatt *et al.*, 2004), we next examined the effect of CTX III on $\triangle\Psi m$ by using the mitochondria-specific dye rhodamine 123 (Scaduto and Grotyohann, 1999). As shown in Figure 5A, there was a dose-dependent decrease in the amount of $\triangle\Psi m$ following treatment with 2 μg/ml

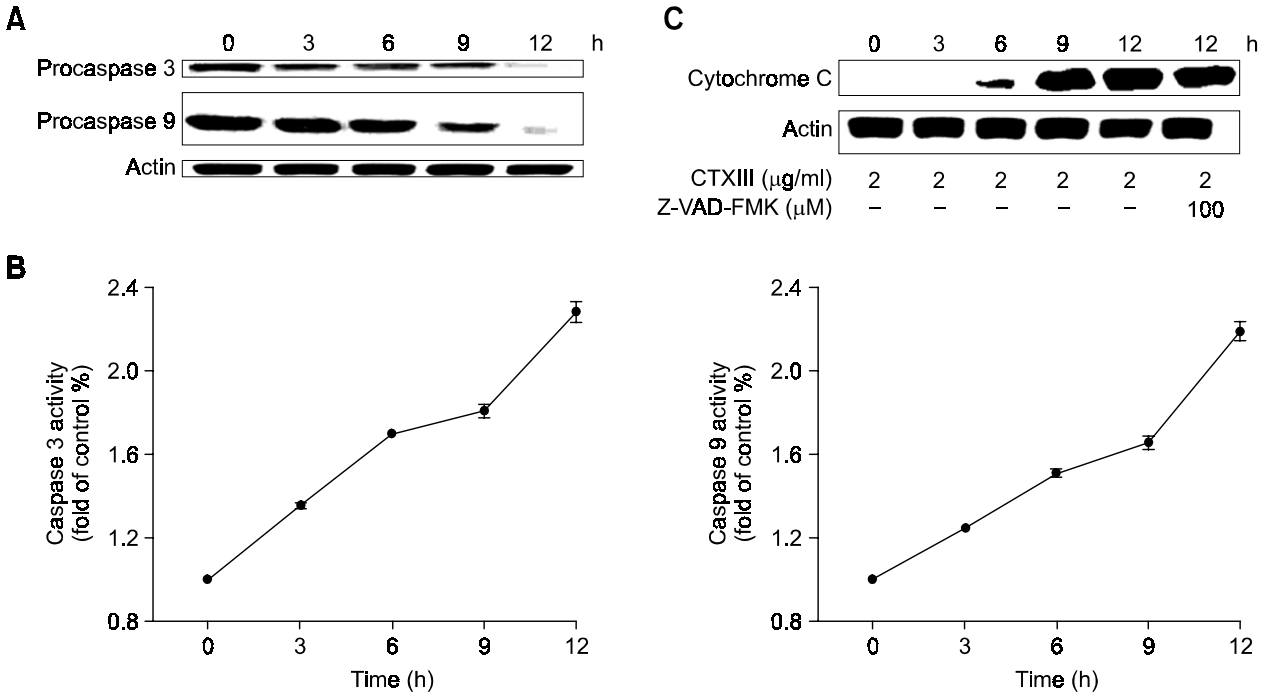

Figure 4. Effects of CTX III on caspase activation and cytochrome c release. (A) Western blot analysis of procaspase-9 and procaspase-3 in CTX III treated K562 cells. Cells were treated with CTX III (2 μg/ml) for the indicated time periods. After treatment, the cytosolic fraction was resolved by SDS-PAGE, transferred onto cellulose membranes, and then probed with specific antibodies. The amount of β-actin was measured as an internal control. Each blot is representative of three independent experiments. (B) Activation of caspases-3 and -9 by CTX III. Cells were treated with 2 μg/ml of CTX III for different time periods. Cell lysates were prepared and the enzymatic activities of the caspases-3 and -9- like proteases were determined by incubation of 50 μg/ml of total protein with colorigenic substrates for 2 h at 37°C. Release of chromophore pNA was monitored spectrophotometrically (405 nm). (C) Accumulation of cytosolic cytochrome c. K562 cells were treated with CTX III (2 μg/ml) for the indicated time periods. For cytochrome c analysis, the cytosolic fractions were separated by SDS-PAGE and transferred onto PVDF membrane. Western blot analysis was performed using a specific antibody for cytochrome c.

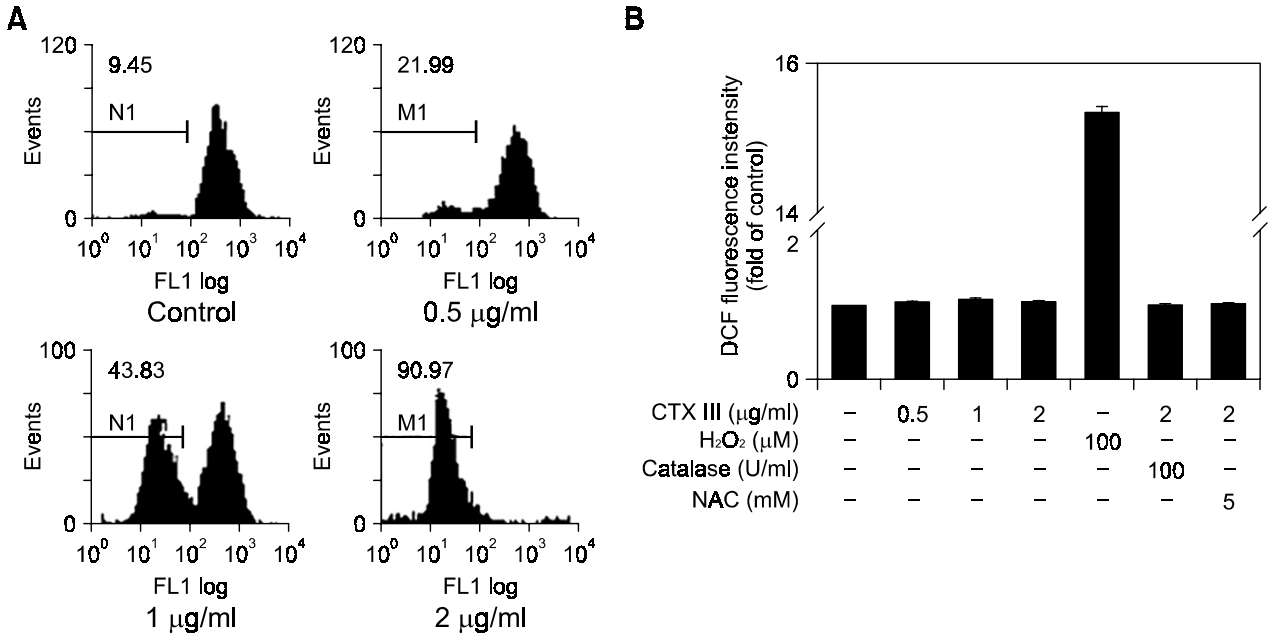

Figure 5. Effects of CTX III on mitochondrial membrane potential and ROS. (A) Effects of CTX III on the mitochondrial membrane potential ($\triangle\Psi$m). Dose-response of $\triangle\Psi$m. Cells were exposed to 2 μg/ml of CTX III for the indicated times, then stained with rhodamine 123 and analyzed immediately by flow cytometry as described in Materials and Methods. The number in M1 indicates the percentage of cells with a reduced $\triangle\Psi$m level. (B) ROS generation is not involved in CTX III-induced apoptosis. Cells (2×10^5 cells/ml) were treated with the indicated concentrations of CTX III for 12 h in a dosage experiment. Harvested cells were then incubated with 20 μM DCFH-DA for 30 min. The intracellular ROS level was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm using a flow cytometer. The relative amounts of intracellular ROS production were expressed as a fluorescence ratio of the treatment to the control. A positive control (H_2O_2-treated cells) was used to monitor the ROS level. Data are expressed as the mean ± SD of three independent experiments.

of CTX III. This result was consistent with an increase in the release of cytochrome c into the cytosol.

ROS generation is not involved in CTX III-induced apoptosis

To investigate whether ROS are involved in CTX III-induced apoptosis, we examined ROS from two aspects. First, we used antioxidants, including NAC (a free radical-scavenging agent) and catalase (an enzyme that hydrolyzes H_2O_2 into H_2O) to investigate their effects on cell viability and the sub G1 fraction. As shown in Figure 2C, neither NAC (5 mM) nor catalase (100 U/ml) had any obvious inhibitory effect on the CTX III-induced cell sub G1 fraction. These results indicate that there is probably no ROS generation in CTX III-treated cells. To confirm this, we directly measured the production of intracellular H_2O_2, which is a final product of intracellular ROS, by using an H2DCF-DA probe. As shown in Figure 5B, CTX III did not significantly increase the intracellular H_2O_2 level in the indicated concentration ranges. These findings indicate that CTX III probably induces apoptosis in K562 cells via a ROS-independent mechanism.

Discussion

Apoptosis is a major form of cell death that is essential for normal development and for maintenance of homeostasis. Current anti-neoplastic therapies, chemotherapy, and radiation therapy are likely to be affected by the apoptotic tendency of cells. Thus, apoptosis has obvious therapeutic implications (Kaufmann and Earnshaw, 2000; Reed, 2001). We found that CTX III-induced K562 cell DNA fragmentation (DNA ladders and sub-G1 formation), PS externalization, and cell death (Trypan blue dye exclusion). CTX III-induced K562 cell death was indicative of typical apoptosis.

Members of the Bcl-2 family of proteins are associated with the mitochondrial membrane and regulate membrane integrity (Adams and Cory, 1998). Some proteins within this family, including Bcl-2 and Bcl-X_L, inhibit apoptosis, while others, such as Bax and Bid, promote apoptosis (Adams and Cory, 1998, Antonsson and Martinou, 2000). Bcl-2 and Bcl-X_L and related anti-apoptotic proteins seem to dimerize with a proapoptotic molecule, e.g. Bax, and modulate the sensitivity of cells to apoptosis (Pastorino *et al.*, 1998). Hence, an alteration in the levels of

anti- and pro-apoptotic Bcl-2 family proteins probably influences apoptosis. CTX III treatment increases the level of the pro-apoptotic protein Bax and decreases the level of the anti-apoptotic protein Bcl-X_L, but not the Bcl-2 and Bid proteins, thereby increasing the Bax/Bcl-2 ratio. This result is consistent with previous observations that apoptosis due to Bax over-expression is caused by a variety of stimuli, including chemotherapeutic agents such as etoposide and paclitaxel (Pastorino *et al.*, 1998). CTX III-mediated cytosolic cytochrome c accumulation was accompanied by up-regulation of Bax (Figure 3A). This result is consistent with many previous reports that regulation of the Bcl-2 proteins and release of cytochrome c are involved in apoptosis (Yang *et al.*, 1997). Mitochondria act as a crossover point between caspase-dependent and -independent apoptotic pathways. The BAX/AIF/Endo G pathway is a major caspase-independent apoptotic cascade (Cande *et al.*, 2004). In this pathway, enhanced Bax expression induces mitochondrial membrane permeabilization, thereby releasing Endo G from the mitochondria to the cytosol and nucleus, with subsequent chromatin condensation and cell death (Figure 3C). Release of these proapoptotic proteins is probably caspase-independent, since a pan-caspase inhibitor did not block CTX III-induced Endo G and cytochrome c, indicating that this effect is upstream of both caspase-3 and caspase-9 (Figure 3C and 4C). Our study demonstrates that CTX III administration induces Endo G release from mitochondria and causes low-scale, nucleosome-size DNA fragmentation (Figure 2A). This is generally caused by the nuclease DFF40/CAD that is activated by both caspase-3 and -7 (Wolf *et al.*, 1999). The latter can be inhibited by the caspase inhibitor Z-VAD-FMK, but this does not prevent DNA laddering in CTX III-treated cells, indicating that another mechanism can cause release of Endo G and subsequent low scale DNA fragmentation (Van Loo *et al.*, 2001). Thus, Endo G represents a pathway that is alternative to DFF40/CAD. However, further studies are needed to systematically explore this possibility.

Several chemotherapeutic and chemopreventive agents have been shown to cause apoptotic cell death through mediation of caspases (Robertson and Orrenius, 2000). We determined whether treatment of K562cells with CTX III resulted in activation of upstream caspase-9 and of downstream caspase-3. After K562 cells were cultured with CTX III (2 μg/ml), caspase-3 and -9 activation in the cytosol were analyzed. As the duration of culture was extended, the activities of caspase -3 and -9 in the cytosol of CTX III-treated cells significantly increased (Figure 4B). One of the early events that initiates apoptosis is release of cytochrome c from the mitochondria into the cytosol (Geen and Reed, 1998; Ravagnan *et al.*, 2002). Consistent with these results, cytochrome c was detected in the cytosol of CTX III-treated K562 cells after 6 h of treatment (Figure 4C). Once released into the cytosol, cytochrome c binds to Apaf-1 and procaspase-9 in the presence of deoxy ATP or dATP to form the apoptosome (Jiang and Wang, 2000). This complex activates caspase -9 which, in turn, cleaves and thereby activates caspase-3 (Shi, 2002). In CTX III-treated cells, release of cytochrome c from the mitochondria was followed by activation of caspases -9 and -3 (Figure 4). These results indicate a linear and specific activation cascade between caspase -9 and caspase -3 in response to cytochrome c release from mitochondria. Cells were pre-treated with Z-VAD-FMK, a caspase inhibitor, causing significant attenuation in the population of apoptotic cells, indicating a caspase-dependent mechanism in cell death due to this toxin. This observation is consistent with a previous report that the caspase inhibitor Z-VAD-FMK had no effect on translocation of cytochrome c to the cytosol, although it was able to block apoptosis in K562 cells (Yang *et al.*, 2005). Another factor contributing to caspase activation in CTX III-treated K562 cells may be decreased IAP expression. Human IAP proteins, including XIAP and survivin, are characterized by the presence of one to three copies of a 70 amino acid motif called the baculoviral inhibitory repeat domain, which bears homology to sequences found in the baculovirus IAP proteins (Deveraux and Reed, 1999). IAPs have been reported to inhibit apoptosis due to their function as direct inhibitors of activated effector caspases, caspase 3, and caspase 7. IAPs are also able to inhibit cytochrome c-induced activation of caspase 9 (Deveraux and Reed, 1999). However, the results of our study demonstrate that the expression levels of XIAP and survivin proteins remained unaltered, indicating that these proteins are not linked to activation of caspases.

Some studies have reported that cancer chemopreventive agents induce apoptosis, in part, through ROS generation and disruption of redox homeostasis (Tong *et al.*, 2004), because addition of exogenous antioxidants, such as NAC or catalase, can inhibit apoptosis (Hou *et al.*, 2004). On the other hand, other studies have reported that apoptosis induced by some anticancer agents is independent of ROS generation (Hou *et al.*, 2004). We found that neither NAC nor catalase blocked the CTX III-induced sub G1 fraction. Direct measurement of the intracellular ROS level with a molecular probe of H2DCFH-DA showed no generation of ROS in CTX III-treated cells. Evidently, apoptosis induced by

CTX III is independent of ROS generation.

A clear picture of the molecular ordering of CTX III-induced events has emerged. Bax up-regulation and Bcl-X_L down-regulation associated with mitochondrial cytochrome c and Endo G release precedes caspase-9-mediated caspase-3 activation and the onset of apoptosis. Treatment with the caspase inhibitor Z-VAD-FMK attenuates CTX III-induced apoptosis. Activation of Bax-dependent and mitochondrial-downstream caspase appears to be an executioner event that leads to CTX III-induced apoptotic cell death in human leukemia K562 cells.

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