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ORIGINAL PAPER

Effects of cardiotoxin III on expression of genes and proteins related to G2/M arrest and apoptosis in K562 cells

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Abstract Cardiotoxin III (CTX III) is a basic polypeptide of 60-amino acid residues isolated from *Naja naja atra* venom, exerts its anti-proliferative activity in human leukemia K562 cells. In the present study, the expression of mRNAs and proteins related to cell cycle and apoptosis in human leukemia K562 cells induced by CTX III was investigated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Flow cytometric analysis revealed that CTX III resulted in G2/M phase arrest in the cell cycle progression, which was associated with a marked decrease in the mRNA and protein expressions of cyclin A, cyclin B1, and Cdk 2, with no detectable changes in the levels of Cdk 1, cyclin D1, and cyclin E. Moreover, the increase in apoptosis was associated with the Bax gene and protein levels significantly increased as treatment durations of CTX III increased, while the Bcl-2 mRNA and protein levels exhibited no changes. We also observed that caspase-9 and caspase-3 genes remained unchanged up to 12 h with 2 μg/ml CTX III. These molecular alterations provide an insight into CTX III-caused growth inhibition, G2/M arrest, and apoptotic death of K562 cells.

Keywords CTX III · G2/M arrest · Apoptosis · K562 cell

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Introduction

Many cytotoxic agents and/or DNA damaging agents arrest the cell cycle at the G1, S or G2/M phase and then induce apoptotic cell death [1–3]. The cell cycle check-point may function to ensure that the cells have time for DNA repair [4, 5]. In recent years, considerable advances have been made in understanding the roles of cyclins, cyclin-dependent kinases (Cdks) in cell cycle progression. This process is regulated by the coordinated action of Cdks in association with their specific regulatory cyclin proteins [5]. G2 to M phase progression is regulated by a number of the Cdk/cyclin family, especially activation of the Cdk 1/cyclin B1 complex is required for transition from G2 to the M phase of the cell cycle [6, 7]. In addition to the cell cycle, apoptosis is an important mode of cell death that occurs in response to a variety of agents including ionizing radiation or anti-cancer chemotherapeutic drugs. The Bcl-2 family including Bcl-2 and Bax contribute to the regulation of apoptosis. In particular, antiapoptotic members of the Bcl-2 family, such as Bcl-2, act to prevent or delay cell death, whereas the pro-apoptotic Bax promotes apoptosis [8]. Recently, several lines of evidence have suggested that the caspase family plays a crucial role in apoptosis [9].

Cardiotoxins (CTXs), a group of highly basic polypeptides of 60-amino acid residues, presenting abundantly in the elapid family of snakes, show very diverse pharmacological functions including hemolysis, cytotoxicity, and depolarization of muscle [10]. Moreover, the toxins showed preferential cytotoxicity toward cancer cells, probably mediated by inhibiting protein kinase C activity or the membrane fusion effect [11–15]. Our previous studies have indicated that CTX III-induced apoptotic cell death in human leukemia K562 cells in a time- and concentration-dependent manner [16], and the CTX III induced apoptosis in K562 cells through an ROS-independent mitochondrial dysfunction pathway [17]. Furthermore, CTX III induced apoptosis has also been established in other cell line, the human colorectal cancer Colo 205 cells [18]. However, the signaling pathways responsible for cell death following CTX III administration remain unclear. To elucidate the mechanism of CTX III, we found that treatment with CTX III on K562 cells resulted in G2/M-growth arrest, presumably involving the concomitant reduction of expression levels of mRNAs and proteins in Cdk 2, cyclin A and cyclin B1. CTX III also caused a marked increase in apoptosis, which was associated with the modulation of Bax, caspase-3 and -9 on human leukemia K562 cells. These molecular alterations provide an insight into CTX III-caused growth inhibition, G2/M arrest, and apoptotic death of K562 cells.

Materials and methods

Chemicals

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), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MD). Antibodies were obtained from the following sources: Bax, Bcl-2, cyclin A, B1, D1, E, Cdk 1, Cdk 2, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse and anti-rabbit Ig G peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Hybond ECL transfer membrane and ECL Western blotting detection kit were obtained from Amersham Life Science (Buckinghamshire, UK).

Cardiotoxins (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. [19]. 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). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO_2.

Flow cytometry analysis

Control and treated cells were harvested, washed in cold PBS, fixed in 70% ethanol and stored at 4°C DNA was treated with RNase A solution (500 unit/ml) at 37°C for 15 min and stained by propidium iodide (50 μg/ml) in 1.12% sodium citrate at room temperature before analysis. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms by the computer program Cell Quest and Mod-Fit.

Western blots

Cells were treated with CTX III for the indicated time points. 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 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonylfluoride), the protein concentration of the supernatant was measured by using the BCA reagents (Pierce, Rockfold, IL, USA). Equal amounts of sample lysate were separated by sodium dodecylsulfate-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. Subsequently, the membrane was washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies. Determinations were performed using enhanced chemiluminescence kits (Amersham, ECL Kits).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNA-zol™ B (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions and quantitated by spectrophotometer. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega Co., Madison,WI). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (Takara Co., Otsu, Japan). Briefly, 50 μl of a reaction mixture including 2.5 units of Taq polymerase (Takara Co.), 5 μl of 10× buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μl of first-strand cDNA, and 25 pmol of each primer (Table 1), was subjected to 35 PCR cycles. The PCR products were analyzed on 1.5% agarose gel.

Statistics

All data are expressed as the mean ± SD. Difference between the treated and the control was analyzed by *t*-test. A probability of $P < 0.05$ was considered significant.

Results

To determine if cell growth inhibition involved cell cycle changes, we examined cell cycle phase distribution by flow cytometry. When cells were treated with various concentrations of CTX III for 12 and 24 h, a similar level of G2/M phase arrest was observed in a dose-and time-dependent patterns (Fig. 1). CTX III treatment induced an accumulation of cells in the G2/M phase of the cell cycle, mainly evident at the concentrations of 1 and 2 μg/ml. For example, 2 μg/ml of CTX III treatment for 24 h resulted in an increase in the percentage of cells in the G2/M phase from 23 to 78%. Concomitant with this increase in the percentage of cells in the G2/M phase was a significant decrease in the percentage of cells in the G0/G1 phase from 30 to 12%. These results suggested that CTX III inhibited the cellular proliferation of K562 cells via G2/M phase arrest of the cell cycle.

Since CTX III arrested K562 cells in the G2/M phase of the cell cycle, we determined the expression levels of cell cycle regulating proteins at the G2/M, such as cyclin A, cyclin B1, Cdk 1, and Cdk 2, were determined by Western blotting (Fig. 2A) and RT-PCR (Fig. 2B). The protein and mRNA levels of Cdk 2, cyclin A, and cyclin B1 were decreased by CTX III treatment, but not in Cdk 1 in a time-dependent manner (Fig. 2). These results suggest that the suppressive effects are caused by downregulating the levels and activities of G2/M phase specific cyclin A, B1, and Cdk 2. However, there was no change in the protein and mRNA levels of cyclin D1 and cyclin E, which regulate the G1 progression and G1/S transition (Fig. 2).

To explore the possible role of Bcl-2 family members in the 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

Table 1 Gene-specific primers used for RT-PCR

Gene name	Primer sequences
Caspase-3	5′-TTTGTTTGTGTGCTTCTGAGCC-3′ 5′ATTCTGTTGCCACCTTTCGG-3′
Caspase-9	5′-GCTCTTCCTTTGTTCATCTCC-3′ 5′-CATCTGGCTCGGGGTTACTGC-3′
Bax	5′-AAAGCTAGCGAGTGTCTCAAGCGC-3′ 5′-TCCCGCCACAAAGATGGTCACG-3′
Bcl-2	5′-AGATGTCCAGCCAGCTGCACCTGAC-3′ 5′-AGATAGGCACCCAGGGTGATGCAAGCFT-3′
Cdk 1	5′-CCATTATTGATCGGTTCATGCAGA-3′ 5′-CTAGTGCAGAATTCAGCTGTGGTA-3′
Cdk 2	5′-GGAGTGGATCCATGGAGAACTTCCAAAAG-3′ 5′-TTGAGAATTCTATCAGAGTCGAAGATGGGG-3′
Cyclin A	5′-CTCCGGTACCAGCCAGTTTGTTTCTC-3′ 5′-TGGCAAGCTTAAGACGCCCAGAGATG-3′
Cyclin B1	5′-CCATTATTGATCGGTTCATGCAGA-3′ 5′CTAGTGGAGAATTCAGCTGTGGTA-3′
Cyclin D1	5′-AATGATCCCGCACGATTTC-3′ 5′CTCAGGTTCAGGCCTTGCA-3′
Cyclin E	5′ATACAGACCCACAGAGACAG3′ 5′TGCCATCCACAGAAATACTT3′
β-actin	5′-CTGTCTGGCGGCACCACCAT-3′ 5′-GCAACTAAGTCATAGTCCGC-3′

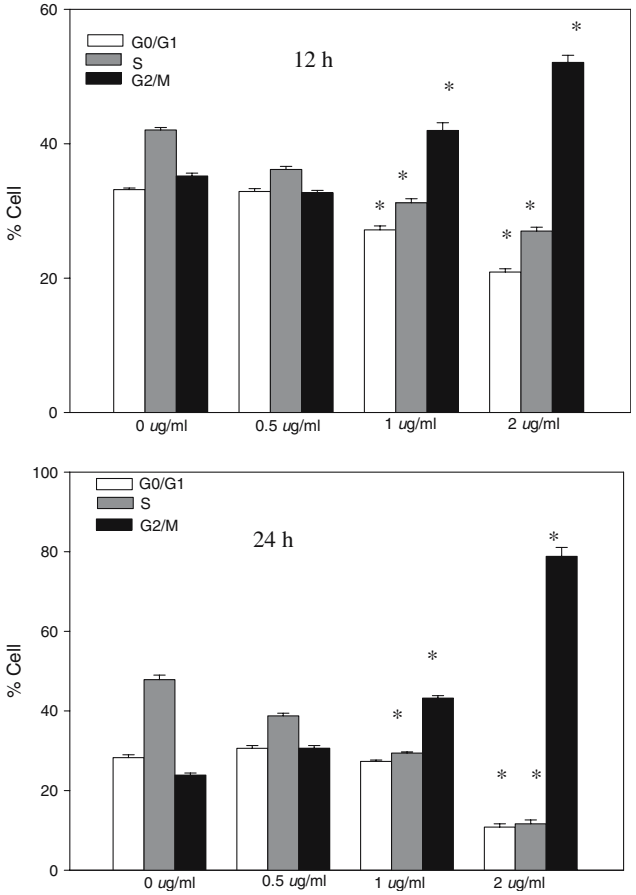

Fig. 1 Effect of CTX III on K562 cell cycle distribution. Cells were treated with the indicated concentrations of CTX III for 12 and 24 h. The cells were then stained with propidium iodide and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). $^{*}P < 0.05$ compared with control

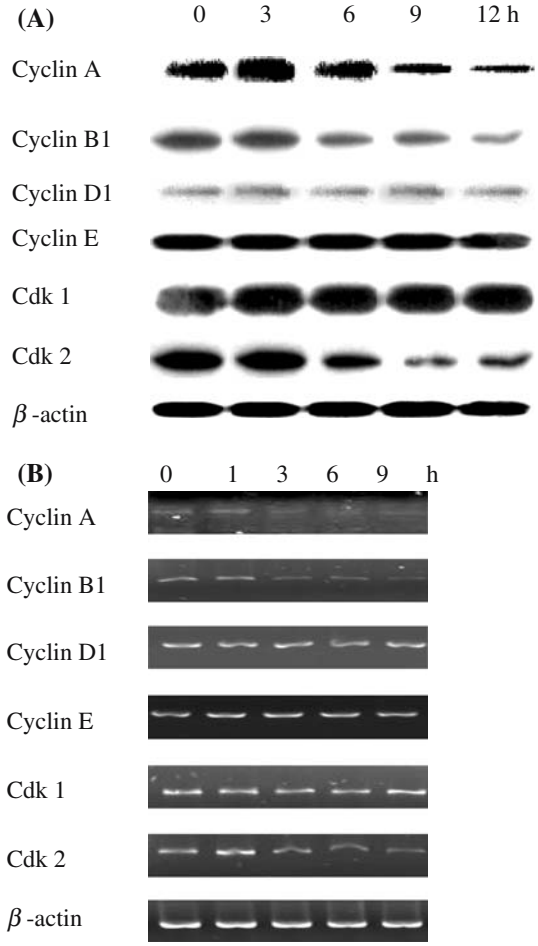

Fig. 2 Effect of CTX III on the levels of cyclins, Cdks and their mRNAs in K562 cells. (**A**) Western blot analysis for the levels of cell cycle regulatory proteins. K562 cells were treated with 2 μg/ml CTX III for 0, 3, 6, 9, and 12 h. Total cell lysates were prepared and 50 μg/well protein was subjected to SDS-PSGE followed by Western blot analysis and chemiluminescent detection. Each antigenic protein was detected by using the respective antibody against Cdk 1, Cdk 2, cyclin A, cyclin B1, cyclin D1, cyclin E, and β-actin. (**B**) RT-PCR analysis of cyclins and Cdks mRNAs in CTX III treated cell. Expression of mRNA in K562 cells treated with 2 μg/ml CTX III for 0, 1, 3, 6, and 9 h. Each blot is representative of three similar experiments

cells to 2 μg/ml of CTX III resulted in upregulating the expression of Bax, while it did not alter the expression levels of Bcl-2 (Fig. 3A). Moreover, the level of Bcl-2 mRNA expression was also unchanged, but the level of Bax mRNA expression was increased (Fig. 3B). The data suggest that CTX III induces apoptosis by the alteration of the ratio of Bcl-2/Bax family protein and gene expressions.

The changes of caspase-3 and -9 mRNA levels in response to the effect with 2 μg/ml CTX III were studied by RT-PCR. Fig. 4 showed that CTX III had no effect on the levels of mRNA transcripts of caspase-3 and -9, suggesting that CTX III regulates this apoptotic factor rather than by regulation of its gene expression.

Discussion

CTX III had time- and dose-dependently antiproliferative effect on K562 cells, but the mechanism is poorly understood. As shown in Fig. 1, CTX III induced a time- and dose-dependent accumulation of K562 cells in the G2/M phase of the cell cycle. G2/M phase accumulation has been observed in cells exposed to DNA damaging agents such as γ-irradiation [20], microtubule-stabilizing agents [21], and topoisomerase inhibitors [22]. To the best of our knowledge, this is the first report describing the mechanism of G2/M phase arrest of CTX III on K562 cells.

Among Cdks that regulate cell cycle progression, Cdk 1and Cdk 2 kinases are activated primarily in association with cyclin A and B1 in the G2/M phase progression. In this study, we found that Cdk 2, cyclin

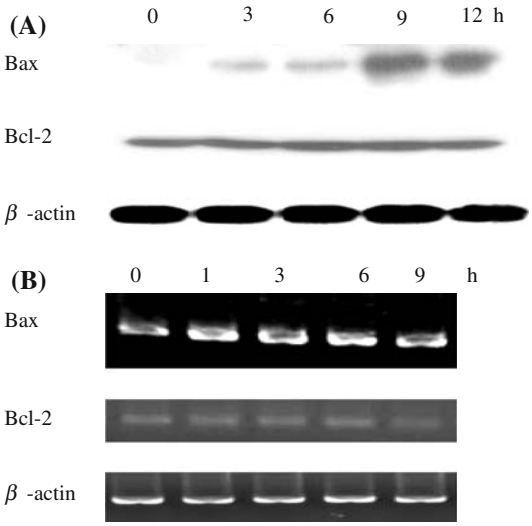

Fig. 3 Effect of CTX III on Bcl-2 family proteins in K562 cells. (**A**) Expressions of Bax and Bcl-2 proteins. After treatment, the fractions were resolved by SDS-PAGE, transferred onto cellulose membranes, then probed with specific antibodies and visualized by chemiluminescence, ECL kit. The amount of β-actin was measured as an internal control. (**B**) RT-PCR analysis of Bcl-2 and Bax mRNAs in CTX III treated cells. Agarose gel electrophoresis of RT-PCR products of Bax, Bcl-2 and β-actin mRNA isolated from K562 cells treated with 2 μg/ml CTX III for 0, 1, 3, 6, and 9 h. Each blot is representative of three similar experiments

A, and cyclin B1 proteins and mRNAs were decreased in a time-dependent manner following the treatment with CTX III, in contrast, the Cdk1 was not changed (Fig. 2). Thus, our data suggest that cell cycle arrest is mediated by limitation of the supply of Cdk2, cyclin A, and cyclin B1, confirming the notion that CTX III induces G2/M arrest. In addition, there was no change in the levels of cyclin D1, and cyclin E, which are activated during the G1 progression and G1/S transition. Thus, it is reasonable to postulate that CTX III

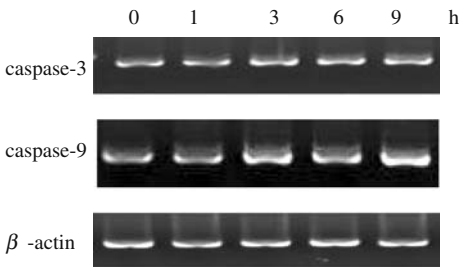

Fig. 4 RT-PCR analysis of caspase-3 and -9 mRNAs in CTX III treated cells. After treatment, expression levels of caspase-9, caspase-3, and β-actin were determined by RT-PCR using specific primers. Cells were treated with 2 μg/ml CTX III for 0, 1, 3, 6, and 9 h. Each blot is representative of three similar experiments

treatment may cause G2/M arrest is brought through signaling cascades that converge to subsequent reduction of cyclin A, cyclinB1 and Cdk 2 protein and mRNA levels.

In addition, CTX III-induced K562 cell DNA fragmentation (DNA ladders and sub-G1 formation) and cell death (Trypan blue dye exclusion) were observed [16, 17]. Based on the above results, 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 regulated its integrity [23]. Bcl-2 and related anti-apoptotic proteins seem to dimerize with a pro-apoptotic molecule, e.g., Bax and modulate the sensitivity of cell to apoptosis [24]. Hence, an alteration in the levels of anti- and pro-apoptotic Bcl-2 family proteins is likely to influence apoptosis. The results of the present study indicate that CTX III treatment increases the level of pro-apoptotic protein and mRNA expression of Bax and no effect on the level of anti-apoptotic protein and mRNA of Bcl-2, thereby increasing the Bax/Bcl-2 ratio (Fig. 3). Therefore, modulation of Bcl-2 and Bax may be a key mechanism underlying apoptosis of K562 cells with CTX III. This is consistent with our previous study that CTX III-induced apoptosis were associated with mitochondrial changes mediated by the Bcl-2 families of proteins, including anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins [18], and Bax accelerated the release of the apoptosis-inducing factor and cytochrome c from the mitochondria, thus activating the caspase cascade [16, 18].

Several chemotherapeutic and chemopreventive agents have been shown to cause apoptotic cell death through mediation of caspases [25]. In the CTX III-treated cells, release of cytochrome c from the mitochondria was followed by activation of caspase -9 and -3 [16, 17]. To determine whether the action of CTX III was mediated through changes in gene expression, RT-PCR analysis was carried out. As shown in Fig. 4, CTX III had no effect on the levels of mRNA transcripts of caspase-3 and -9, indicating that CTX III regulating apoptotic factor at the protein level rather than by regulation of its gene expression.

In summary, these results suggest that CTX III inhibits the proliferation of K562 cells via G2/M arrest in association with the reduction of Cdk 2, cyclin A and cyclin B1 activities and a clear picture of the molecular ordering of CTX III-induced events has emerged, starting with the Bax up-regulation, caspase-9 and -3 activation and the onset of apoptosis. Thus, these findings provide important new insights into the possible molecular mechanisms of the anti-cancer activity of CTX III.

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