J. Toxicol. Pub. Health
Vol. 23, No. 3, pp. 215-221 (2007)

Official Journal of
Korean Society of Toxicology

Available online at http://www.toxmut.or.kr

Requirement of Reactive Oxygen Species Generation in Apoptosis of MCF-7 Human Breast Carcinoma Cells Induced by Sanguinarine

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Received August 1, 2007; Accepted August 23, 2007

Although sanguinarine, a benzophenanthridine alkaloid, possesses anti-cancer properties against several cancer cell lines, the molecular mechanisms by which it inhibits cell growth and induces apoptosis have not been clearly understood. In order to further explore the critical events leading to apoptosis in sanguinarine-treated MCF-7 human breast carcinoma cells, the following effects of sanguinarine on components of the mitochondrial apoptotic pathway were examined: generation of reactive oxygen species (ROS), alteration of the mitochondrial membrane potential (MMP), and the expression changes of Bcl-2 family proteins. We show that sanguinarine-induced apoptosis is accompanied by the generation of intracellular ROS and disruption of MMP as well as an increase in pro-apoptotic Bax expression and a decrease of anti-apoptotic Bcl-2 and Bcl-xL expression. The quenching of ROS generation with N-acetyl-L-cysteine, the ROS scavenger, protected the sanguinarine-elicited ROS generation, mitochondrial dysfunction, modulation of Bcl-2 family proteins, and apoptosis. Based on these results, we propose that the cellular ROS generation plays a pivotal role in the initiation of sanguinarine-triggered apoptotic death.

Key words: Sanguinarine, Reactive oxygen species, Mitochondrial membrane potential, Apoptosis.

INTRODUCTION

Sanguinarine is a benzophenanthridine alkaloid that derived from the root of *Sanguinaria canadensis* L., *Chelidonium majus* L. and the seeds of *Argemone mexicana* L. (Mahady and Beecher, 1994; Vavreckova *et al.*, 1996). This compound widely used in toothpaste and mouth washes to suppress dental plaque formation and reduce gingival inflammation (Laster and Lobene, 1990; Mandel, 1998), possibly via its antioxidant, antimicrobial and anti-inflammatory effect. Recently, several studies have indicated that sanguinarine, at micromolar concentration, inhibits the growth of various human cancer cell lines that are associated with cell cycle arrest and stimulation of apoptotic cell death (Adhami *et al.*, 2000, 2003, 2004; Debiton *et al.*, 2003; Ding *et al.*, 2002; Weerasinghe *et al.*, 2001a, b, 2006; Hussain *et al.*, 2007; Chang *et al.*, 2007; Han *et al.*, 2007). Because sanguinarine is relatively non-toxic to normal cells, an important implication of these findings is that the compound may be useful for cancer therapy. However, the molecular mechanisms of its action have not been completely understood.

Apoptosis, a programmed cell death, plays an important role in regulating the number of cells during development, in homeostatic cell turnover in adults, and in many other settings (Makin and Dive, 2001; Ghobrial *et al.*, 2005). However, most cancer cells can block apoptosis, which allows them to survive despite the genetic and morphologic transformations. In general, apoptosis can be initiated in two ways: by an extrinsic pathway or

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Abbreviations: AMPs, apoptogenic mitochondrial proteins; DAPI, 4,6-diamidino-2-phenylindile; DCFH-DA, 2,7 dichlorofluorescein diacetate; ECL, enhanced chemiluminescence; EtBr, ethidium bromide; IAPs, inhibitor of apoptosis proteins; JC-1, 5,5 V, 6,6 V-tetrachloro-1,1 V,3,3 V-tetraethyl-imidacarbocyanine iodide; MMP, mitochondrial membrane potential; NAC, N-acetyl-L-cysteine; PBS, phosphate-buffered saline; PI, propidium iodide; PTPC, permeability transition pore complex; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel

an intrinsic pathway. The intrinsic pathway is initiated by the mitochondria, while the extrinsic pathway is initiated by cell surface receptors (Chowdhury *et al.*, 2006; Fulda and Debatin, 2006). Mitochondria are the major generators of ATP by oxidative phosphorylation and mitochondria-mediated apoptosis occurs in response to a wide range of stimuli. Reactive oxygen species (ROS) are generated in and around mitochondria and they are buffered by antioxidants. ROS may not only arise from endogenous sources but also from exogenous sources (Thannickal and Fanburg, 2000; Hagen and Vidal-Puig, 2002). ROS production is increased in cancer cells, which lead to alterations of proliferative and apoptotic control by constitutive activation of multiple redox-sensitive targets, including components of signaling cascades as well as transcription factors (Suh *et al.*, 1999). Thus, constitutively elevated levels of cellular oxidative stress may represent a redox vulnerability of malignancy that can be targeted by chemotherapeutic intervention using redox modulators, and both anti- and prooxidant agents have been shown to exert anticancer activity (Thannickal and Fanburg, 2000; Hagen and Vidal-Puig, 2002). Two previous studies suggested that sanguinarine increases intracellular ROS levels and results in apoptosis (Hussain *et al.*, 2007; Chang *et al.*, 2007). Although many studies have shown that sanguinarine has an effect in various tumor cells, currently the role of mitochondrial functional changes in the response of breast cancer cells to sanguinarine has not yet been explored.

We hypothesized that sanguinarine would induce functional changes in the mitochondria in association with ROS generation in the course of apoptosis induction in breast cancer cells. To test this hypothesis, we evaluated the effect of sanguinarine on mitochondrial membrane potential (MMP), ROS, and apoptosis using human breast carcinoma MCF-7 cells. Our results indicate that ROS generation is required for the apoptosis induced by sanguinarine in MCF-7 cells.

MATERIALS AND METHODS

Reagents. Sanguinarine (13-methyl (1,3) benzodioxolo [5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium), propidium iodide (PI), 4,6-diamidino-2-phenylindole (DAPI), ethidium bromide, 2,7 dichlorofluorescein diacetate (DCFH-DA) and 5,5 V, 6,6 V-tetrachloro-1,1 V,3,3 V-tetraethylimidacarbocyanine iodide (JC-1) were obtained from Sigma (St Louis, MO, USA). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the peroxidase-labeled donkey anti-rabbit immunoglobulin, peroxidase-labeled sheep anti-mouse immunoglobulin and an enhanced chemiluminescence (ECL) kit was purchased from Amersham (Arlington Heights, IL, USA). RPMI 1640 medium was purchased from Invitrogen Corp. (Carlsbad, CA, USA) and fetal bovine serum (FBS) was purchased from GIBCO-BRL (Gaithersburg, MD, USA). All other chemicals not specifically cited here were purchased from Sigma.

Cell culture and growth inhibition study. MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with heat-treated 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. Sanguinarine was dissolved in methanol as a stock solution at a 10 mM concentration and was stored in aliquots at -20°C. For growth inhibition analysis, cells were seeded and exposed to various concentrations of sanguinarine for 48 h. The cells were trypsinized, washed with phosphate-buffered saline (PBS) and the viable cells were scored with a hemocytometer through exclusion of trypan blue.

Nuclear staining with DAPI. After treating the cells with sanguinarine, the cells were harvested, washed in PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with DAPI solution for 10 min at room temperature. The nuclear morphology of the cells was examined by fluorescence microscopy (Carl Zeiss, Germany).

Flow cytometry analysis for measurement of sub-G1 phase. The cells were harvested and fixed in ice-cold 70% ethanol and stored at 4°C. Prior to analysis, the cells were washed once again with PBS, suspended in 1 ml of a cold PI solution containing 100 μg/mL RNase A, 50 μg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer (FACS Caliber, Becton Dikinson, San Jose, CA) and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence.

DNA fragmentation assay. The cells were treated with different sanguinarine concentrations for 48 h and lysed on ice in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min. The lysates were vortexed and cleared by centrifugation at 10,000 g for 20 min. The fragmented DNA in the supernatant was extracted using an equal volume of neutral phenol : chloroform : isoamylalcohol

(25 : 24 : 1, v/v/v) and analyzed electrophoretically on 1% agarose gel containing EtBr.

Protein extraction and Western blotting. The cells were harvested and lysed. The protein concentrations were measured using a Bio Rad protein assay (BioRad Lab., Hercules, CA, USA) according to the manufacturer's instructions. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gels and transferred by electroblotting to a nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody and visualized by ECL according to the recommended procedure.

Measurement of intracellular ROS. ROS production was monitored using the stable non-polar dye DCFH-DA that readily diffuses into cells (Cathcart *et al.*, 1983). The cells were seeded in 24-well plates and incubated in the absence or presence of sanguinarine for different periods of time. Later the cells were incubated with 10 μM DCFH-DA for 30 min. The ROS production in cells was monitored by flow cytometer using cell quest software (Beckton Dickinson).

Assessment of mitochondrial membrane potential (MMP, $\Delta\Psi_m$). To measure the MMP, the dual-emission potential-sensitive probe JC-1 was used. After treatment with sanguinarine, 5×10^5 cells were collected, stained with 10 μM JC-1 at 37°C for 20 min and then analyzed using flow cytometer (Becton Dickinson).

RESULTS

Inhibition of cell viability by sanguinarine in MCF-7 cells. In order to determine if sanguinarine decreases the cell viability, MCF-7 cells were stimulated with various concentrations of sanguinarine for 48 h, and the cell

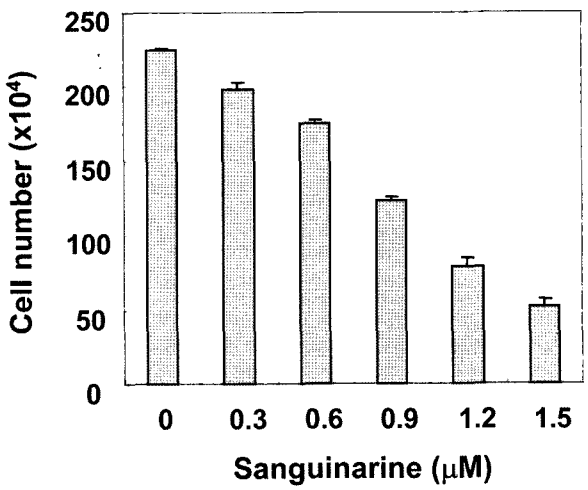

Fig. 1. Inhibition of MCF-7 cells proliferation by sanguinarine. Cells cells were plated at 1×10^5 cells per 60-mm plate, and incubated for 24 h. The cells were treated with various concentrations of sanguinarine for 48 h and the viable cell number was determined by hemocytometer counts of trypan blue-excluding cells. The data shown are mean ± SD of three independent experiments.

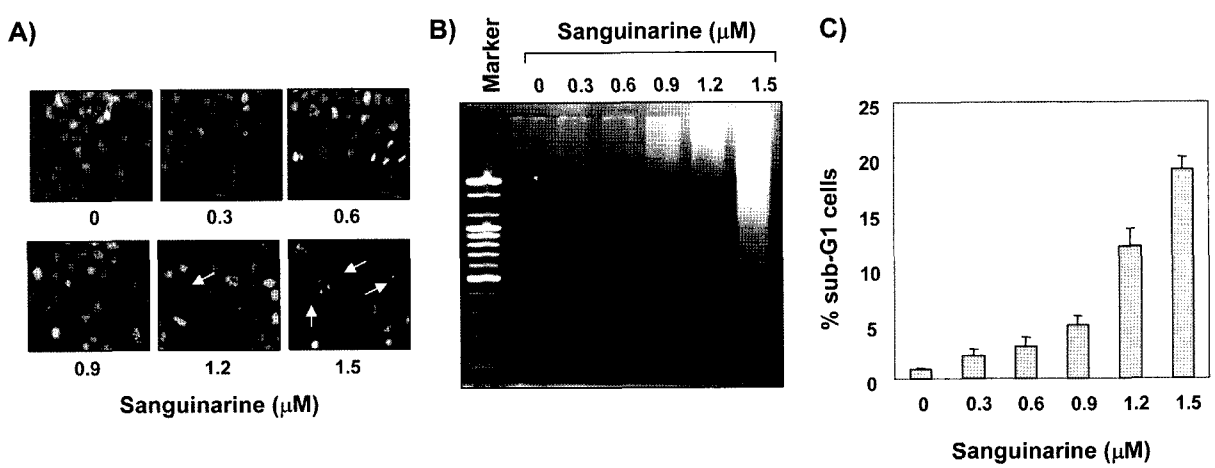

Fig. 2. Induction of apoptosis by sanguinarine in MCF-7 cells. (A) After treated with sanguinarine for 48 h, the cells were fixed, stained with DAPI and the nuclear morphology was photographed with a fluorescence using blue filter (365 nM). Magnification, × 400. (B) For the analysis of DNA fragmentation, the genomic DNA was extracted and analyzed on a 1.0% agarose gel. (C) To quantify the degree of apoptosis induced by sanguinarine, the cells were evaluated for sub-G1 DNA content represent the fractions undergoing apoptotic DNA degradation using a flow cytometer. Each point represents the average of three independent experiments.

viability was measured by a hemocytometer counting. As shown in Fig. 1, the sanguinarine significantly inhibited the cell viability in a concentration-dependent manner. After 48 h treatment, sanguinarine inhibited the cell viability at 0.9 μM and 1.5 μM by approximately 50% and 75% compared with the control, respectively.

Induction of apoptosis by sanguinarine in MCF-7 cells. Further experiments using fluorescence microscopy, agarose gel electrophoresis and flow cytometry analyses were carried out to determine if the inhibitory effect of sanguinarine on the cell viability is the result of apoptotic cell death. Morphological analysis with DAPI staining showed nuclei with chromatin condensation and the formation of apoptotic bodies in the cells cultured with sanguinarine in a concentration-dependent manner. On the other hand, very few were observed in the control culture (Fig. 2A). Agarose gel electrophoresis indicated that sanguinarine treatment induced the progressive accumulation of fragmented DNA, which appeared as a typical ladder pattern of DNA fragmentation due to internucleosomal cleavage associated with apoptosis (Fig. 2B). Thus, the degree of apoptosis was determined by analyzing the amount of sub-G1 DNA in the cells treated with sanguinarine using flow cytometer. As shown in Fig. 2C, the addition of sanguinarine resulted in the increased accumulation of cells in the sub-G1 phase in a similar manner to that observed with the sanguinarine-induced loss of cell viability, formation of apoptotic bodies, and accumulation of extranuclear fragmented DNA. This suggests that MCF-7 cells may undergo apoptosis after exposure to sanguinarine, and there is a good correlation between the extent of apoptosis and the inhibition of growth.

Effects of sanguinarine on ROS generation and MMP. It is well established that apoptosis induced by some agents is associated with the perturbation of mitochondrial functions. Therefore, we investigated changes in mitochondrial function in response to sanguinarine treatment. Using the fluorescent dyes DCFH-DA and JC-1, it was assessed the changes in ROS production and MMP following sanguinarine treatments in MCF-7 cells. Elevating the time of sanguinarine treatment to 1 h and 4 h evidently increased the ROS production to 98.8-fold and 147.2 fold of control, as revealed by DCFH-DA fluorescence (Fig. 3A). And, treatment with sanguinarine also induced mitochondrial membrane hyperpolarization in a concentration-dependent manner (Fig. 3B). In order to show that the loss of MMP is associated with the generation of ROS, the cells were pretreated with 10 mM N-acetyl-L-cysteine (NAC), a commonly used

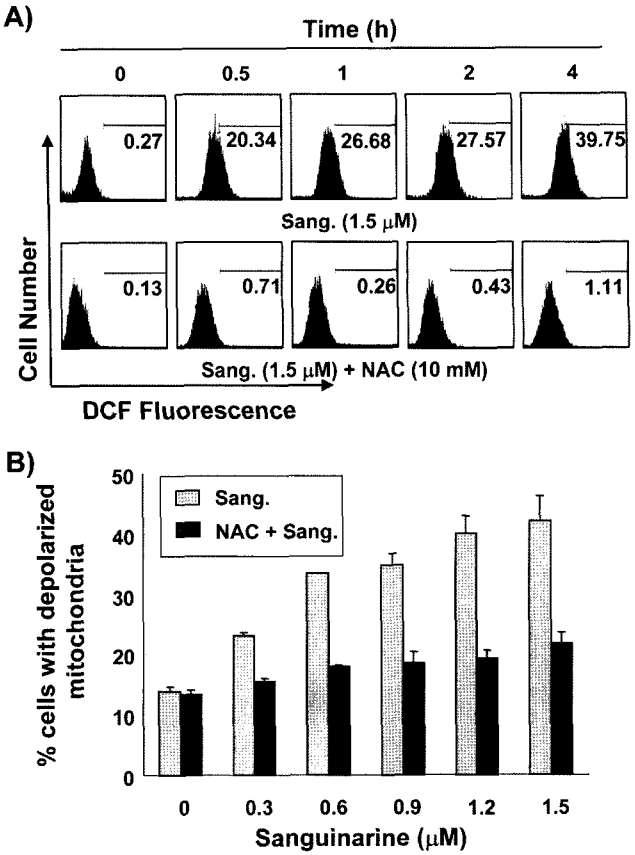

Fig. 3. ROS generation and mitochondrial membrane hyperpolarization in sanguinarine-treated MCF-7 cells. (A) The cells were treated with NAC (10 mM) for 1 h before challenge with 1.5 μM sanguinarine for indicated times, and then stained with DCFH-DA at 37°C for 30 min. At each time point, the fluorescence intensity was measured by flow cytometer. Data are means from two representative experiments. (B) The cells under the same conditions as (A) were stained with JC-1 at 37°C for 20 min at different time points. Mean JC-1 fluorescence intensity detected by flow cytometer. Data are means ± SD from representative experiments performed at least 3 times.

quencher of ROS, for 1 h, followed by treatment with sanguinarine. As shown in Fig. 3A and 3B, pre-treatment with NAC effectively blocked sanguinarine-induced ROS generation and loss of MMP (Fig. 3).

Effects of sanguinarine on the levels of Bcl-2 and IAPs family proteins. To elucidate mechanisms underlying sanguinarine-induced apoptosis, we investigated the levels of Bcl-2 and the inhibitor of apoptosis proteins (IAPs) family proteins by Western blotting. When MCF-7 cells were treated with sanguinarine a clear increase in pro-apoptotic Bax protein expression was observed in sanguinarine-treated cells (Fig. 4). In the case of anti-apoptotic proteins such as Bcl-2 and Bcl-

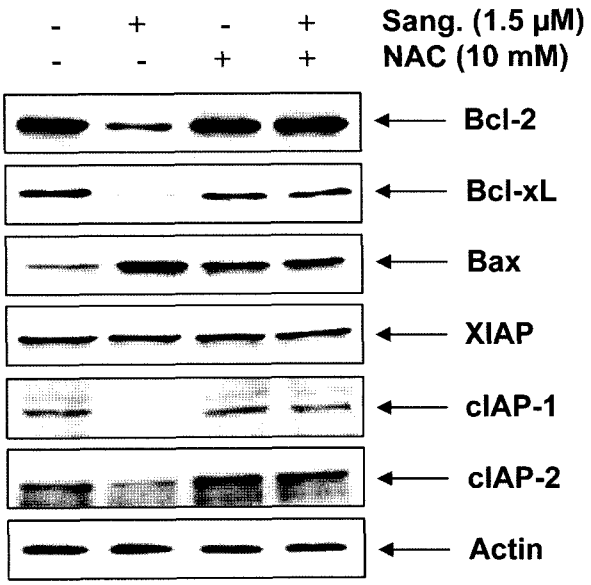

Fig. 4. Prevention of the alteration of apoptosis-related proteins by quenching of ROS generation in sanguinarine-treated MCF-7 cells. The cells were incubated with or without 10 mM NAC for 1 h before being treated with sanguinarine for 48 h, and the cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

xL, there was a marked down-regulation observed in cells treated with sanguinarine. The levels of IAPs family proteins such as cIAP-1 and cIAP-2 after sanguinarine treatment were also down-regulated in cells treated with PTX-2. However, sanguinarine did not significantly affect the levels of XIAP proteins in MCF-7 cells (Fig. 4). These results indicated that sanguinarine-induced apoptosis of MCF-7 cells were associated with modulation of the Bcl-2 and IAPs family proteins.

Sanguinarine-induced apoptosis was associated with the generation of ROS. In order to show that the generation of ROS is a key step in the sanguinarine-induced apoptotic pathway, MCF-7 cells were pretreated with NAC for 1 h, followed by treatment with various concentrations of sanguinarine for 48 h. At 10 mM, NAC did not have effect on apoptosis, but the presence of NAC almost completely suppressed sanguinarine-induced apoptosis, as evidenced by a near-complete reversal of the percentage of sub-G1 cells (Fig. 5A). Furthermore, the blocking of the generation of ROS by pretreatment of the cells with NAC markedly prevented the sanguinarine-induced DNA fragmentation (Fig. 5B). These findings indicate that we could use NAC to demonstrate

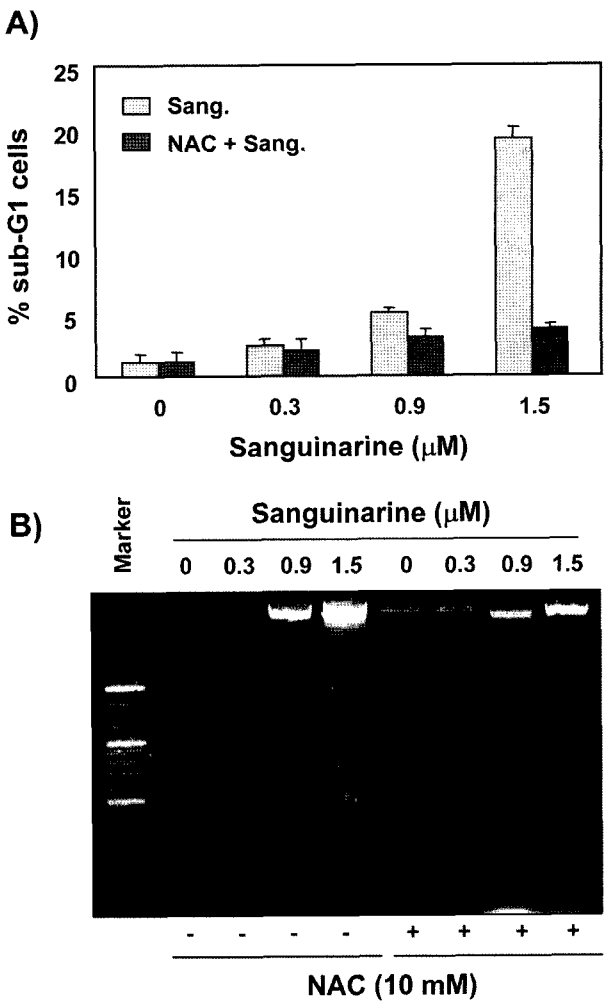

Fig. 5. Inhibition of sanguinarine-induced apoptosis by quenching of ROS generation in MCF-7 cells. (A) The cells were treated with NAC (10 mM) for 1 h before a challenge with various concentrations of sanguinarine for 48 h and collected. The cells were evaluated for sub-G1 DNA content using a flow cytometer. Data are reported as mean ± SD of three independent experiments. (B) The genomic DNA was extracted from cells under the same conditions as (A) and analyzed on a 1.0% agarose gel.

whether ROS is essential for sanguinarine-induced apoptosis. Furthermore, NAC significantly blocked not only up-regulation of pro-apoptotic Bax protein but also down-regulation of Bcl-2, Bcl-xL, cIAP-1 and cIAP-2 proteins (Fig. 3). These results clearly show that an increase in ROS is required for sanguinarine-induced apoptosis in MCF-7 cells.

DISCUSSION

The study was carried out to evaluate the mechanism of action of sanguinarine induced cytotoxicity in

cultured MCF-7 breast carcinoma cells. In the present study, we demonstrated that sanguinarine induced apoptosis in MCF-7 cells through the generation of ROS. This interpretation was based on the increase of ROS generation in the sanguinarine-treated MCF-7 cells and on the significant protection against apoptosis exerted by NAC, the ROS scavenger. Our results corroborated and extended the findings of Hussain *et al.* (2007) and Chang *et al.* (2007) on the induction of oxygen free radicals, mitochondria damage, and apoptosis in lymphoma and nasopharyngeal cancer cells, respectively.

It is clear that changes of mitochondrial membrane potential are associated with apoptosis. Both hyperpolarization and depolarization have previously been observed (Poppe *et al.*, 2001) however, mitochondrial depolarization in association with apoptosis appears to be more common. During apoptosis, the permeability transition pore complex (PTPC) is formed in mitochondrial membrane to affect MMP (Tsujimoto and Shimizu, 2002). The apoptogenic mitochondrial proteins (AMPs) including cytochrome *c* are released from the intermembrane space into cytoplasm. In the cytosol, cytochrome *c* binds to Apaf-1 and triggers Apaf-mediated caspase-9 activation. Activation of caspase-9 activates caspase-3 and other caspases (Chowdhury *et al.*, 2006; Fulda and Debatin, 2006). The activated caspase family members hydrolyze the cytoplasmic proteins and activate DNase, which degrades genomic DNA nucleosomes.

Caspase activation is often regulated by various cellular factors, including members of IAPs and Bcl-2 family members. IAPs family has been reported to exert anti-apoptotic effects due to their function as direct inhibitors of activated caspases. Therefore, down-regulation of IAPs relieves the triggering block of proapoptotic signaling and the execution caspases, thus activating cell death (Deveraux and Reed, 1999). Bcl-2 family proteins play a central role in the regulation of mitochondria-mediated apoptosis. In some models they are regulated at the expression level, while in others only mitochondrial translocations seen (Makin and Dive, 2001; Ghobrial *et al.*, 2005; Fulda and Debatin, 2006). For example, Bax, a pro-apoptotic member, normally resides in the cytosol. After exposure to apoptotic stimuli, Bax translocates into mitochondria, undergoes a conformational change and is associated with the voltage-dependent anion channel (VDAC). The VDAC-Bax complex promotes pore formation and the release of AMPs (Tsujimoto and Shimizu, 2002). In our study, a significant fall in MMP, down-regulation of Bcl-2 members such as Bcl-2 and Bcl-xL as well as IAPs family proteins such as cIAP-1 and cIAP-2, and up-regulation of Bax proteins were observed in cells exposed to sanguinarine as compared to control cells.

Mitochondria are the rich source of ROS, which are toxic byproducts of aerobic cells and play an important role in cell proliferation, aging, and cancer development. An excessive amount of ROS can lead to cell death by apoptosis or by necrosis. Excessive mitochondrial ROS generation can cause the PTPC to open and regulate the release of AMPs and apoptosis (Skulachev, 2006). In many cases, mitochondria-produced ROS are involved in apoptosis and the importance of chemotherapy-induced change in redox status to signal apoptosis and regulate the apoptosis effector is emerging (Thannickal and Fanburg, 2000; Hagen and Vidal-Puig, 2002; Ozben, 2007). For further confirmation, the role of ROS production in sanguinarine toxicity, NAC, a general free radical scavenger, was tested to clarify the nature of ROS and its relation to sanguinarine toxicity. Interestingly, the increasing accumulation of intracellular ROS was found in sanguinarine-treated cells and NAC reduced intracellular ROS as well as preventing the loss of MMP. Moreover, the blocking of the ROS generation by pretreatment of the cells with NAC also prevented the sanguinarine-induced DNA fragmentation and the apoptosis, indicating that ROS production and mitochondrial dysfunction are therefore the possible contributing factors of sanguinarine toxicity. In addition, NAC significantly blocked the changes of Bcl-2 and IAPs family proteins in sanguinarine-treated MCF-7 cells. This indicates that the modulation of Bcl-2 and IAPs family proteins by sanguinarine was associated with ROS production.

In conclusion, sanguinarine induced oxidative stress and mitochondrial damage, which then caused apoptosis in breast carcinoma MCF-7 cells. The mechanism of apoptosis induction by sanguinarine involved the generation of ROS. The NAC, a ROS blocking agent, significantly prevented apoptosis induced by sanguinarine. Although, in this paper we only roughly assessed whether ROS was involved in the apoptosis induced by sanguinarine, the present results may help to understand the mechanisms for the anti-cancer activity of sanguinarine.

ACKNOWLEDGEMENTS

This research was supported by a grant (M2007-11) from Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs & Fisheries, Republic of Korea.

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