Apoptosis Induction of Human Lung Carcinoma Cells by Chan Su (Venenum Bufonis) Through Activation of Caspases

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Abstract
Chan Su is a traditional Chinese medicine prepared from the dried white secretion of the auricular and skin glands of toads, and has been used as an oriental drug for the treatment of a number of diseases, including cancer. In this study, the potential of Chan Su (skin of Venenum Bufonis) to induce apoptosis in human lung carcinoma A549 cells was investigated. Treatment of A549 cells with skin of Venenum Bufonis resulted in the inhibition of cell growth and viability and the induction of apoptosis, which was shown by trypan blue counts, MTT assay, DAPI staining and flow cytometry analysis. The increase in apoptosis that was induced by skin of Venenum Bufonis was correlated with down-regulation of anti-apoptotic Bcl-2 expression, up-regulation of pro-apoptotic Fas ligand and death receptor 4, and a decrease in the mitochondrial membrane potential. Skin of Venenum Bufonis treatment induced the proteolytic activation of caspases and a concomitant degradation of poly(ADP-ribose)-polymerase and β-catenin protein. Cleavage of Bid and a down-regulation of the inhibitor of apoptosis family proteins were also observed in skin of Venenum Bufonis-treated A549 cells. Data from this study indicates that SVB induces the apoptosis of A549 cells through a signaling cascade of death receptor-mediated extrinsic and mitochondria-mediated intrinsic caspase pathways.

1. Introduction
Apoptosis is a tightly regulated process characterized by cell shrinkage, plasma membrane blebbing, and chromatin condensation that is consistent with DNA cleavage into ladders [1,2]. Apoptosis plays an important role in developmental processes, maintenance of homeostasis, and elimination of...
damaged cells. Several gene products have been demonstrated to be critical in the regulation of apoptosis. For instance, caspases, a group of cysteine proteases, play key roles in apoptosis [3,4]. Caspases are synthesized as proenzymes, which are activated by the cleaving of the prodomain at a specific aspartic acid cleaving site [5,6]. Caspase activation is often regulated by various cellular factors, including members of the Bcl-2 family and/or death receptor-related gene products [6–9]. However, most cancer cells can block apoptosis, which allows them to survive despite genetic and morphologic transformations. Many recent studies have shown that many chemopreventive and/or chemotherapeutic agents can cause tumor cell death via the induction of apoptosis, which is the preferred method of managing cancer. Therefore, the induction of apoptotic cell death is an important mechanism in the anticancer properties of many drugs.

In Chinese traditional medicine, amphibian skin extract has been used for the alleviation of many human ailments. Toads, particularly members of the genus Bufo, are identified as a particularly useful and readily available source of granular gland secretions, which commonly contain biogenic amines, bufodienolides, alkaloids and steroids, peptides and proteins [10,11]. Chan Su (Venenum Bufonis) is the dried white venom prepared from the skin secretions of a toad (Bufo bufo gargarizans Cantor), which has been used in China and other Asian countries for centuries to treat a number of diseases, including cancer, arrhythmia and other heart diseases [12,13]. In several previous reports, Chan Su has been shown to be potent in the treatment of various cancer cells in vitro by induction of cell cycle arrest and apoptosis [14–21]. Recently, we also reported that Chan Su is significant in inducing the apoptosis against human bladder carcinoma cells, which was mediated by an increase in Bax expression, decrease in Bcl-2 expression and a proteolytic activation of caspase-3 and -9 [22]. The apoptotic effects of Chan Su were also associated with a specific inhibition of COX-2 expression and prostaglandin E2 production [22]. However, the molecular mechanisms of its anti-proliferative action on malignant cell growth are not completely understood.

In the present study, to further evaluate the cytotoxic effects of Chan Su in cancer cells, the effects of an extract of Chan Su (skin of Venenum Bufonis, SVB) on apoptosis in human lung carcinoma A549 cells were investigated. We found that SVB-induced-apoptosis is accompanied by modulation of the death receptor system, Bcl-2 family members, mitochondrial dysfunction and activation of caspases.

2. Materials and Methods

2.1. Cell culture, SVB treatment, and cell growth assay

Human lung carcinoma A549 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humified environment with 5% CO2 at 37°C. The SVB was obtained from Dunsan Oriental Hospital (Daejeon, South Korea). One hundred grams of SVB was washed with distilled water, and then boiled in 1 L water at 80°C for 2.5 hours. Solid particles and aggregates were removed by centrifugation at 3,000 g for 30 minutes and the supernatants were lyophilized. Finally, 21.7 g lyophilized SVB were obtained and used in this experiment. The lyophilized extract was stored at −20°C until used. For growth inhibition studies, cells were cultured in the absence and presence of variable concentrations of SVB for 24 hours. The cells were trypsinized, washed with phosphate-buffered saline (PBS), and the viable cells were scored with a hemocytometer through the exclusion of trypan blue. Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes [23]. For morphological studies, the cells were treated with SVB for 24 hours and directly photographed with an inverted microscope (Carl Zeiss, Germany).

2.2. Nuclear staining with DAPI

For evidence of apoptosis, morphological changes of nuclei were visualized following DNA staining using the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI, Sigma). After incubation for 24 hours with SVB, cells were fixed with 3.7% paraformaldehyde for 20 minutes at room temperature, and washed with PBS. Cells were then stained with 2.5 μg/mL DAPI solution for 10 minutes at room temperature. The cells were then washed twice with PBS and stained nuclei were observed using fluorescence microscopy (Carl Zeiss).

2.3. DNA flow cytometric analysis

After treatment with SVB, cells were harvested, washed twice with ice-cold PBS, fixed with 75% ethanol at 4°C for 30 minutes, and stained using
a DNA staining kit (CycleTEST PLUS Kit, Becton Dickinson, San Jose, CA, USA) with propidium iodide (PI). DNA content at sub-G1 phase was then determined by flow cytometry (FACSCalibur) and analyzed by Cell Quest software (Becton Dickinson).

2.4. Mitochondrial membrane potential (MMP, $\Delta \Psi_m$) assay

To measure the MMP, 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Calbiochem, San Diego, CA, USA), a dual-emission fluorescent dye, was used. JC-1 is internalized and concentrated by respiring mitochondria and can reflect changes in MMP in living cells. There are two excitation wavelengths, 527 nm (green) for the monomer form and 590 nm (red) for the JC-1 aggregate form. Briefly, the cells were collected and incubated with 10 $\mu$M JC-1 for 30 minutes at 37°C. Cells were then washed once with cold PBS and analyzed using a DNA flow cytometer.

2.5. Protein extraction and western blot analysis

Cells were collected with ice-cold PBS, and immediately lysed with lysis buffer (20 mM sucrose, 1 mM EDTA, 20 $\mu$M Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM MgCl$_2$, containing protease inhibitors (5 $\mu$g/mL pepstatin A, 10 $\mu$g/mL leupeptin, and 2 $\mu$g/mL aprotinin). Protein concentrations were determined using Bio-Rad protein assay kits (Bio-Rad, Hercules, CA, USA). After normalization, total proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) via electroblotting. The membrane was blocked with 5% skim milk, and incubated with the primary antibodies and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies. The target proteins were visualized by an enhanced chemiluminescence (ECL, Thermo scientific, Rockford, IL, USA) detection system. Antibodies were purchased from Calbiochem (San Diego, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham (Arlington Heights, IL, USA).

2.6. Assay of caspases activity

The enzymatic activity of caspases that had been induced by SVB was recorded using colorimetric assay kits based on the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA). Briefly, cells were lysed in a lysis buffer for 30 minutes on an ice bath. The lysed cells were centrifuged at 14,000 rpm for 10 minutes, and 100 $\mu$g protein was incubated with 50 $\mu$L of reaction buffer and 5 $\mu$L of colorimetric tetrapeptides, Asp-Glu-Val-Asp (DEVD)-p-nitroanilide (pNA) for caspase-3, Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8 and Leu-Glu-His-Asp (LEHD)-pNA for caspase-9 at 37°C for 2 hours. The optical density of the reaction mixture was measured by changes in absorbance at 405 nm using a VersaMax tunable microplate reader (Molecular Devices, Palo Alto, CA, USA).

2.7. Statistical analysis

The data is expressed as means±SD. Statistical comparisons were performed using one-way ANOVA followed by a Fisher test. Significant differences between the groups were determined using an unpaired Student’s t-test. A p value <0.05 was considered significant.

3. Results

3.1. Growth inhibition and apoptosis induction by SVB in A549 cells

To determine if SVB decreases cell growth, A549 cells were stimulated with various concentrations of SVB and the cell number and viability were then measured by trypan blue exclusion method and MTT assay. As shown in Figure 1, treatment with SVB significantly inhibited the viability and proliferation of cells, and these effects occurred in a concentration-dependent manner. In addition, direct observation by inverted microscopy revealed that numerous morphological changes occurred in cells treated with SVB (Figure 2A). In particular, cell shrinkage, cytoplasm condensation, and formation of cytoplasmic filaments appeared in a concentration-dependent manner after SVB treatment. To elucidate whether SVB inhibits the proliferation of A549 cells by inducing apoptosis, cells treated with SVB were examined after DAPI staining. The control cells displayed an intact nuclear structure; whereas, cells treated with SVB had chromosomal condensation and formation of apoptotic bodies (Figure 2B). Thus, the degree of apoptosis was determined by analyzing the amount of sub-G1 DNA in A549 cells that were treated with SVB using flow cytometry. As shown in Figure 2C, the treatment of A549 cells with SVB resulted in the increased accumulation of cells in the sub-G1 phase. Taken together, these results suggest that A549 cells can undergo apoptosis after being exposed to SVB. In addition, these results revealed a good correlation between the extent of apoptosis and the inhibition of growth.
Apoptosis induction of human lung carcinoma cells

3.2. Effects of SVB on the levels of apoptosis-related genes in A549 cells

To investigate the apoptotic pathways activated by SVB, we used Western blot analysis to measure the expression of the death receptors and corresponding pro-apoptotic ligands (Fas, Fasl, TRAIL, DR4 and DR5), as well as the expression of the Bcl-2 family members (Bcl-2, Bcl-XL, Bax, Bad and Bid). As shown in Figure 3, the protein levels of

Figure 1  Inhibition of the cell growth by SVB in A549 human lung carcinoma cells. The cells were plated at 4×10⁴ cells per 60mm plate, and incubated for 24 hours. The cells were treated with varying concentrations of SVB for 24 hours and cell viability and growth inhibition were, respectively, measured by the metabolic-dye-based MTT assay (A) and hemocytometer counts of trypan blue-excluding cells (B). Data is expressed as mean±SD of three independent experiments. Statistical analysis was carried out by t-test (*, p<0.05).

Figure 2  Induction of apoptosis by SVB treatment in A549 cells. (A and B) After treating the cells with SVB for 24 hours, they were observed using an inverted microscope (A, magnification, ×200) or were stained with DAPI for 10 minutes, washed with PBS, and then photographed with a fluorescence microscope using a blue filter (B, magnification, ×400). (C) To quantify the degree of apoptosis induced by SVB, the cells were evaluated for sub-G1 DNA content using a flow cytometer. This represents the fractions undergoing apoptotic DNA degradation. Each point represents the average of two independent experiments.
FasL and DR4 expression were markedly increased in the SVB-treated A549 cells, whereas the levels of anti-apoptotic Bcl-2 expression were significantly inhibited in response to the SVB treatment in a concentration-dependent manner. However, the levels of Fas, DR5, TRAIL, Bcl-xL, Bax and Bad expression were relatively unchanged in response to SVB treatment. Additionally, Western blot analyses revealed that treatment with SVB induced the cleavage of Bid (tBid), a BH3-only pro-apoptotic member of the Bcl-2 family. This data indicates that both the extrinsic and intrinsic pathways might be involved in SVB-induced apoptosis in A549 cells. The expression levels in the SVB-treated cells were also examined to determine if SVB induces A549 cell death through a change in the expression of the inhibitor of apoptosis (IAP) family proteins, which binds the caspases and leads to caspase inactivation for an anti-apoptotic effect. As shown in Figure 3, SVB induced a concentration-dependent decrease in the expression levels of the XIAP, cIAP-1 and cIAP-2 proteins.

3.3. Loss of MMP and activation of caspases by SVB

The role of the mitochondria in SVB-induced apoptosis was further investigated by examining the effect of SVB on the MMP and caspase activity in A549 cells. Exposure of A549 cells to various concentrations of SVB led to a significant reduction in the level of MMP, and this reduction occurred in a dose-dependent manner (Figure 4). In addition, SVB treatment resulted in down-regulation of pro-caspase-3, -8 and -9 (Figure 5A). To further quantify the proteolytic activation of the caspases, the concentration of protein in the lysates of cells treated with SVB was normalized and then assayed for their caspase-3, -8 and -9 activity using DEVD-pNA, IETD-pNA and LEHD-pNA, respectively, as substrates. As shown in Figure 5B,
treatment with SVB increased the activity of the caspases in a concentration-dependent manner. Subsequent Western blot analysis revealed that progressive proteolytic cleavage of the poly(ADP-ribose) polymerase (PARP) and β-catenin proteins, which are downstream targets of the activated caspase-3 [24,25], occurred in A549 cells that were treated with SVB, and that this cleavage was induced in a concentration-dependent manner [Figure 5A]. These results indicate that activation of caspases plays an important role in SVB-induced apoptosis in A549 cells.

4. Discussion

In recent years, various studies have been conducted that show how Chan Su may impinge on cellular signaling pathways and often may recruit them to induce apoptosis in cancer cells [14–22]. However, the mechanisms responsible for the apoptotic effects of Chan Su have not yet been determined. In the present study, SVB was shown to significantly inhibit proliferation and cell viability and induced mitochondrial damage and apoptosis in human lung carcinoma A549 cells.

Mitochondria have recently been suggested as a possible target for anti-cancer drug induced apoptosis. Since the discovery of Bcl-2, several mechanisms for the anti-apoptotic properties of this protein have been proposed [6–8]. The anti-apoptotic function of Bcl-2 against pro-apoptotic Bax may be explained by its ability to control several key steps involved in

![Graph showing loss of mitochondrial membrane potential (%)](image)

**Figure 4** Loss of MMP by SVB treatment of A549 cells. After being treated with SVB for 24 hours, the cells were stained with JC-1 and then incubated at 37°C for 20 minutes, after which the mean JC-1 fluorescence intensity was detected using a flow cytometer. Data represent the mean±SD of representative experiments performed at least three times. The significance was determined by a Student’s t-test (*, p<0.05 vs. untreated control).

![Graph showing activity of caspases (fold-induction)](image)

**Figure 5** Activation of caspases and the degradation of the PARP and β-catenin protein by SVB treatment in A549 cells. (A) After 24 hours incubation with SVB, the cells were lysed. The cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-PARP and anti-β-catenin antibodies. The proteins were visualized using an ECL detection system. Actin was used as the internal control. (B) The cell lysates from the cells grown under the same conditions as (A) were assayed for in vitro caspase-3, caspase-8 and caspase-9 activity using the substrates DEVD-pNA, IETD-pNA and LEHD-pNA, respectively. The concentrations of the fluorescent products released were measured. Data are expressed as mean±SD of three independent experiments. For statistical analysis, t-test was performed (*, p<0.05).
death signaling. Bcl-2 can form ion channels in mitochondrial membranes, which may allow it to control apoptosis by influencing the permeability of the intracellular membranes and release of substances into the cytoplasm [4,8,9]. These observations demonstrated that the Bcl-2 family significantly regulates apoptosis either as an activator (Bax) or as an inhibitor (Bcl-2); therefore, it has been suggested that the Bax/Bcl-2 ratio is a key factor in regulation of the apoptotic process [4–9]. The results of this study indicate that SVB treatment results in the induction of apoptosis in A549 cells, which was confirmed by characteristic morphological changes, chromatin condensation and increases in the number of sub-G1 cells (Figure 2). Therefore, we first evaluated the effects of SVB on the levels of proteins in the Bcl-2 family in A549 cells to test the mechanism by which SVB-induced apoptosis occurs. Our results demonstrate that SVB did not alter expression levels of pro-apoptotic Bax but did markedly down-regulate the expression of anti-apoptotic Bcl-2 (Figure 3), which was associated with a reduction in the level of MMP (Figure 4). The data indicates that the SVB increased Bax/Bcl-2 ratio and induced mitochondrial dysfunction lead to apoptosis in A549 cells.

The results of many studies have suggested that reduction of Bcl-2 or induction of Bax leads to the release of cytochrome c from the mitochondria into the cytosol, as well as an increase in the activity of caspases [5,6]. The caspases are a family of proteins that are one of the main executors of the apoptotic process in both the extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) apoptotic pathways. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens. Caspase signaling is initiated and propagated by proteolytic autocatalysis, as well as the cleavage of downstream caspases and substrates [3–6]. The death receptor pathway is initiated by binding of ligands to the death receptors on the cell surface, which then activates caspase-8 and then cleavage of Bid (tBid), which further activates a series of caspase cascades, resulting in apoptotic cell death [7–9]. However, changes in the mitochondrial integrity in response to a broad range of physical and chemical stimuli can trigger the intrinsic pathway of apoptosis [2,6,9,26]. Once in the cytosol, cytochrome c can activate caspase-9, which in turn cleaves and activates the key executioner, caspase-3, which is either partially or totally responsible for the proteolytic cleavage of many key proteins including PARP and β-catenin that are important for cell viability but also serves as markers of apoptosis when cleaved [24,25]. Further studies have demonstrated that SVB increased the levels of FasL and DR4 expression, key regulators of the extrinsic apoptotic pathway, and the enzymatic activity of both extrinsic and intrinsic caspase cascades such as caspase-8 and -9. In addition, the decreased levels of IAP family proteins and down regulation of length Bid expression were observed in SVB-treated A549 cells (Figure 5). These results indicate that SVB may activate the extrinsic apoptotic pathway and increase mitochondrial dysfunction, which, in turn, results in the activation of caspase-9, leading to the activation of caspase-3, which is also associated with the degradation of caspase-3 target proteins.

In summary, our results indicate that SVB strongly suppresses the proliferation of A549 cells by induction of apoptosis through activation of the mitochondrial mediated-intrinsic caspase pathway along with the death receptor-mediated extrinsic pathway. Although further studies are needed, we believe that SVB is a promising candidate for cancer chemoprevention and/or chemotherapy.

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References


