Inhibitory Effect of Snake Venom on Colon Cancer Cell Growth Through Induction of Death Receptor Dependent Apoptosis

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I. Introduction

Colon cancer is a major malignancy with a worldwide cumulative incidence rate of 9.4% and the second leading cause of cancer\(^1\). It develops in the cecum, colon and rectum\(^2\), which are confined within the intestinal wall is treatable by enucleation of the local lesions. However, in the advanced colon cancer metastasizing into the deeper regions or other organ such as liver, surgical treatment alone has the limitations\(^2\). Although chemotherapy with anticancer drugs, such as 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin is usually adopted for inhibiting the cancer growth and prolonging survival, it remains unsatisfactory due to current status of comparatively poor cancer drug development and cancer stem cell related highly chemoresistance in colon cancer\(^3\). Anti-cancer drug treatment generally results in apoptosis, programmed cell death through the activation of caspase cascade systems via triggering Death Receptor (DR) dependent apoptotic pathway at the cell surface, activating caspase-8 or cytochrome C dependent pathway at the mitochondria, characterized by loss of mitochondrial permeability transition, release of mitochondrial cytochrome C into the cytoplasm, consecutive activation of caspase-8 or -9, and caspase-3\(^7\). However, it is usual for the activated apoptosis related pathway to represent only some of the characteristics of the above classical apoptotic pathways in anti-cancer agents, depending on cancer cell type\(^10\). According to the previous reports\(^12\), acquired resistance to chemotherapeutics is closely related to the lower apoptosis rate. Therefore, a new remedy enhancing it for colon cancer is urgently needed.

A snake venom toxin (SVT) from *Vipera lebetina turanica*, is a group of basic peptides, and important factor V activator made up of 236 amino acids with six disulfide bonds formed by twelve cysteins\(^14\). A few researchers revealed that SVT exerts its effect on cellular proteins such as Bax, Bcl-2, caspases in the classical apoptotic way, and that SVT inhibits cancer growth through the induction in cancers such as neuroblastoma and prostate cancer\(^15-17\). However, experiments demonstrating the molecular mechanisms of the anti-cancer effects of SVT in colon cancer cells have not been reported. Thus, in the present study, I investigated apoptosis related anti-cancer effects of SVT and confirmed whether increase of DR expression, cell cycle arrest, and inhibition of activity of NF-κB signal molecules or STAT3 pathway in the human colon cancer, HCT116 cells.

II. Materials and methods

A. Materials

SVT from *Vipera lebetina turanica* was purchased from Sigma Chemical Co. (Saint Louis, USA). DR3, DR4, and DR6 siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All of the secondary antibodies such as Bax, Bcl-2, caspase-3, cleaved caspase-3, caspase-8, caspase-9, cleaved caspase-9, p50, p65, PARP and STAT3,
used in Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma unless otherwise stated.

B. Cell culture

The HCT116 colon cancer cells were obtained from ATCC (American Type Culture Collection, Rockville, MD). Colon cells were cultured in RPMI-1640 medium (Life Technologies Inc., Gaithersburg, MD) supplement with 10% FCS (Fetal Calf Serum; Collaborative Biomedical Products, Bedford, MA) and antibiotics, penicillin/streptomycin (100 unit/ml, Bioproducts, Walkersville, MD). Cell cultures were then maintained at 37°C in a humidified atmosphere of 5% CO₂.

C. Cell viability assay

To determine the cell number, HCT116 colon cancer cells or CCD112 normal colon cells were plated in 24-well plates (5 × 10⁴ cells/well), and subconfluent cells were subsequently treated with SVT (0.1, 0.5 and 1 µg/ml) for 24 hr. After treatment, cells were trypsinized and pelleted by centrifugation for 5 min at 1,500 rpm, resuspended in 5 ml of phosphate-buffered saline (PBS), and 0.1 ml of 0.2% trypan blue was added to the cancer cell suspension in each of the solutions (0.9 ml each). Subsequently, a drop of suspension was placed into a Neubauer chamber and the living cancer cells and normal colon cells were counted. Cells that showed signs of staining were considered to be dead, whereas those that excluded trypan blue were considered viable. Each assay was carried out in triplicate.

D. Cell cycle analysis

To examine the effect of SVT on cell cycle distribution of asynchronous populations of HCT116 colon cancer cells, replicative DNA synthesis and DNA content were analyzed using bivariate flow cytometric analysis. Cells were harvested by trypsin-EDTA release and fixed in ice-cold 70% ethanol. At least 1–2 hr before flow cytometric analysis, cells were resuspended in a 1 ml aliquot of modified Vindelov’s DNA staining solution (10 µg/ml RNase A and 5 µg/ml propidium iodide in phosphate-buffered saline). Flow cytometric analysis was performed with flow cytometry system (FACS Calibur-S System, BD Bioscience, SanJose, CA). Cells in the G1, S, and G2/M phase of the cell cycle were determined with Modfit LT (Verity House Software, Top-sham, ME).

E. Reverse transcription (RT)-PCR

Total RNAs were isolated from cultured cells using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s manual. The RNA pellet obtained in the final step was dissolved in 30 µl of sterile diethylpyrocarbonate (DEPC)-treated water, and its concentration was determined using a UV spectrophotometer at 260 nm. RNA was kept in DEPC-treated water at −70 until use. Reverse transcription was performed using High Capacity RNA-to-cDNA Kit (AB). PCR amplifications were then carried out with the following primers.

<table>
<thead>
<tr>
<th>Death receptor</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>TNFR 1</td>
<td>5'-ACC AAG TGC CAC AAA GGA AC-3'</td>
<td>5'-CTG CAA TTG AAG CAC TGG AA-3'</td>
</tr>
<tr>
<td>TNFR 2</td>
<td>5'-CTC AGG AGC ATG GGG ATA AA-3'</td>
<td>5'-AGC CAG CCA GTC TGA CAT CT-3'</td>
</tr>
<tr>
<td>DR 3</td>
<td>5'-ATG GCG ATG GCT GCG TGT CCT G-3</td>
<td>5'-AGC GCC TCC TGG GTC TCG GGG TAG-3</td>
</tr>
<tr>
<td>DR 4</td>
<td>5'-ACT TTG GTT GTT CCG TTG CTG TTG-3</td>
<td>5'-GCC TTT CCA TTT GCT GCT CA-3</td>
</tr>
<tr>
<td>DR 5</td>
<td>5'-TGG AAC AAG GGG GAC AGA AGC-3</td>
<td>5'-GCA GCG CAA GCA GAA AAG GAG-3</td>
</tr>
</tbody>
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F. Western blot analysis

Western blot analysis was done as described previously. The membrane was incubated for 2 hr at room temperature with specific antibodies: rabbit polyclonal for caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-8, caspase-9, cleaved caspase-9, PARP, Cleaved PARP, Bcl-2 (1:1,000 dilution, Cell Signaling Technology, Inc., Beverly, MA), p-Stat3, Bax, p65, p50 (1:500 dilution, Santa Cruz Biotechnology, Inc.), and mouse monoclonal Stat 3 (1:500 dilution, Santa Cruz Biotechnology, Inc.). The blot was then incubated with the corresponding conjugated anti-rabbit and anti-mouse immunoglobulin G-horseradish peroxidase (1:2,000 dilutions, Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected with the ECL Western blotting detection system.

Transfection. HCT116 colon cancer cells (3 × 10⁴ cells per well) were plated in 24-well plates and transiently transfected with siRNA, using a mixture of siRNA and the WelFect-EXPLUS reagent and OPTIMEN, according to the manufacturer’s specification (WelGENE, Seoul, Korea). The transfected cells were treated with 0.1, 0.5 and 1 μg/ml SVT for 24 hr.

DR3 siRNA seq. 5’-GAAGCCCUAAGUACGGUATT
DR4 siRNA seq. 5’-CUCUGAUGCUGUUCUUUGATT
DR6 siRNA seq. 5’-GCCUUCUAGUGUGAUGAATT

G. Apoptosis evaluation

HCT116 colon cancer cells (2.5 × 10⁵ cells/well) were cultured on 8-chamber slides. After colon cancer cells were transfected with siRNA, the cells were treated with SVT (5 μg/ml). The cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1 hr at room temperature. Membrane was permeabilized by exposure to 0.1% Triton X-100 in phosphate-buffered saline for 5 min at room temperature. TdT-mediated dUTP nick and labeling (TUNEL) assays were performed by using the in situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. For 4'-6-Diamidino-2-phenyl indole (DAPI) staining, slides were incubated for 15 min at room temperature in the dark with mounting medium for fluorescence containing DAPI (Vector Laboratories, Inc., Burlingame, CA). The cells were then observed through a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany).

H. Data analysis

The data were analyzed using the GraphPad Prism 4 ver. 4.03 software (GraphPad Software, La Jolla, CA). Data are presented as mean ± SD. The differences in all data were assessed by one-way analysis of variance (ANOVA). When the P value in the ANOVA test indicated statistical significance, the differences were assessed by the Dunnett’s test. A value of P < 0.05 was considered to be statistically significant.

III. Results

A. Effect of SVT toxin on cell growth in HCT116 colon cancer cells

To assess the inhibitory effect of SVT on cell growth of HCT116 colon cancer cells, we analyzed cell viability by direct cell counting. The cells were treated with several concentrations of SVT (0.1, 0.5, and 1 μg/ml) for 24 hr. As shown in Fig. 1, SVT inhibited cell proliferation of HCT116 colon cancer cells in a concentration-dependent manner, whereas it didn’t exert an influence on CCD112 normal colon cell viability.

B. Expression of death receptors in HCT116 colon cancer cells by SVT

Apoptosis can be induced by stimulation of DRs
Inhibitory Effect of Snake Venom on Colon Cancer Cell Growth Through Induction of Death Receptor …

Fig. 1. Effect of SVT on cell viability in HCT116 colon cancer cells
Concentration-dependent effect of SVT on the cell viability assay in HCT116 or CCD112. After treatment of SVT (0.1, 0.5 and 1 µg/ml) for 24 hr, the cells were harvested by trypsinization and stained with 0.2% trypan blue. Relative cell survival rate was determined by counting live and dead cells. The results were expressed as a percentage of viable cells. Columns, means of three experiments, with triplicates of each experiment; bars, SD.

expression. Therefore, to investigate expression of DRs in cancer cells undergoing apoptotic cell death, we performed RT-PCR analysis. RT-PCR analysis showed that SVT treatment increased TNF-R1, DR4 and DR5 mRNA levels in a concentration dependent manner, but TNF-R2, DR3 expression levels were not changed by SVT in HCT116 cells (Fig. 2).

C. Cell cycle arrest at the G0/G1 and G2/M phase
To evaluate whether arrest of the cells in specific cell cycle could be related with cell death, I analyzed cell cycle after treatment of SVT. As increasing the concentration of SVT over 0.1 µg/ml, the number of cells distributed in S phase was decreased significantly compared to the cells in other phases. The percentage of the cells in the S phase was about 35% in the untreated both cells, but the number was gradually decreased up to about 17% by the treatment of SVT (1 µg/ml), whereas, SVT arrested the cell in both the G0/G1 phase and the G2/M phase (Fig.3). The percentage of the cells presented in the G0/G1 phase was 47.7% (control), 43.3% (0.1 µg/ml), 51.4% (0.5 µg/ml)
Fig. 3. Cell cycle analysis of HCT116 colon cancer cells treated with various doses of SVT DNA content was analyzed by flow cytometry as described in the Materials and Methods. HCT116 colon cancer cells were treated with 0 to 1 μg/ml SVT for 24 hr. Each panel is representative of three similar experiments with triplicates.

and 54.3% (1 μg/ml) in colon cancer cell, respectively. The percentage of the cells presented in the G2/M phase was 18.8% (control), 23.1% (0.1 μg/ml), 29.1% (0.5 μg/ml) and 29.3% (1 μg/ml) in colon cancer cell, respectively.

Moreover, to investigate the influence of SVT upon cell cycle regulatory proteins such as cyclin B1, cyclin D, cyclin E, I performed western blot analysis, showing that SVT increased the cyclin B1, cyclin D and cyclin E expressions concentration dependently in HCT116 colon cancer cells (Fig. 3).

D. Apoptotic cell death by SVT

To determine the inhibition of cell growth by SVT was due to the induction of apoptotic cell death, we evaluated the changes in the chromatin morphology of cells by using DAPI staining followed by TUNEL staining assays, and then the double labeled cells were analyzed by fluorescence microscope. Conversely well with cell growth inhibition, DAPI-stained TUNEL-positive cells were significantly increased in SVT treated cells dose-dependently. The treatment of SVT over 1 μg/ml resulted in about 60-70% induction of apoptotic cell death in HCT116 colon cancer cells (Fig. 4).

E. Effect of SVT on the expression of apoptotic regulatory proteins

To figure out the relationship between the induction of apoptotic cell death and the expression of their regulatory proteins by SVT, expression of apoptotic cell death related proteins was investigated by Western blots. The expression of
Expression of apoptosis regulatory proteins was determined using Western blot analysis. The HCT116 colon cancer cells were treated with different concentrations of SVT (0.1, 0.5, and 1 µg/㎖) for 24 hr. Equal amounts of total proteins (50 µg/lane) were subjected to 12% or 8% SDS-PAGE. Expression of PARP, caspase-3, and -9, Bax, Bcl-2, and β-actin were detected by Western blotting using specific antibodies. β-actin protein here was used as an internal control. Each band is representative for three experiments.

F. Effect of SVT on the activation of NF-κB signal molecules and STAT3

The Phosphorylation of IκB in a NF-κB signal pathway and STAT3 is associated with proliferation and maintenance of tumors. Thus, activation of NF-κB signal molecules and STAT3 pathway was investigated. We found that the expression of NF-κB signal molecules and p-STAT3 were not changed by SVT in HCT116 colon cancer cells (Fig. 6).

IV. Discussion

The central and noteworthy finding in this study is the confirmation of anti-cancer efficacy of SVT against colon cancer, HCT116 cells through induction of apoptosis via increase of DR expressions, cell cycle arrest, impact on cellular proteins including PARP, caspases-3, -9, Bax and Bcl-2 in the classical apoptotic pathway.

Apoptosis is the process of programmed cell death that majorly involves in anti-cancer effects in cancers, and it is also considered to be a promising mechanism to overcome the acquired chemoresistance[2,13,18]. According to several researches, it was found that natural agents-induced apoptotic...
cell death could be selectively triggered by increase of DR expression\(^{19-23}\). In other words, expression of DR induces activation of caspase-8 and then make the apoptosis going through the activation of pro-apoptotic proteins including caspases-9 and -3, as well as Bax in the DR dependent apoptotic systems\(^{24,25}\). In the present study, Similar with the above, my data represented that SVT inhibited cell proliferation of HCT116 colon cancer cells in a concentration-dependent manner through about 60-70% induction of apoptotic cell death, and that it also increased TNF-R1, DR4 and DR5 mRNA levels dose-dependently, triggering the subsequent increase of the expression of pro-apoptotic proteins including PARP, Bax, cleaved form of caspase-3, -8, and -9, and concomitant decrease of that of anti-apoptotic protein, Bcl-2.

Meanwhile, the induction of apoptosis is also closely related with the regulation of cell cycle progression in which cyclin dependent kinases (CDK), CDK inhibitor and cyclins are involved. CDK inhibitors are tumor suppressor proteins down-regulating the cell cycle progression by binding active CDK-cyclin complexes and thereby inhibiting their kinase activities\(^{26,27}\). Cyclin D is rate limiting for the progression of the cell cycle from the G1 phase to the S phase, during which DNA replication occurs\(^{28}\). Cyclin E, an essential cyclin that is expressed in late G1 and early S phase, regulates the phosphorylation of many proteins that are important for cell proliferation\(^{29}\). cyclin B1 is likely to be most important in mitotic regulation the target of multiple mitotic checkpoints, which is related with a mechanism by which the p53 tumor suppressor inhibits G2/M transition\(^{30,32}\).

Cell-cycle checkpoints, receptor collectives are responsible for the control of cell-cycle phase progression, which is emerging as a novel target of anti-cancer agents for increasing cytotoxicity of chemotherapy\(^{30-33}\). G0/G1 or G2/M arrest have also been associated with enhanced apoptosis, for defects in the G1 or G2/M checkpoint may lead a cancer cell to enhanced apoptosis through correcting abnormal proliferation of damaged cells or inducing mitosis of them\(^{34-37}\).

According to Sauter et al’s report\(^{38}\), down-regulation of cyclin D through antisense treatment led to the apoptosis of melanoma cells and caused tumor shrinkage of xenotransplants in nude mice. Moreover, Son et al. demonstrated that SVT-induced G0/G1 and G2/M phase arrest, accompanied by the reduction of cell distribution into the S phase, is a possible mechanism for cell growth inhibition leading to apoptotic cell death. In this study, consistent with the above, SVT caused a weak G0/G1 cell cycle arrest and a strong G2/M arrest in the colon cancer. However, expression of G1 or G2/M phase regulating protein cyclin D, E or cyclin B1 was upregulated in the colon cancer HCT116 cells, unlike the above prostate cancer cells.

In addition, activated NF-\(\kappa\)B or STAT3 plays important roles in cell growth, proliferation, survival, differentiation, apoptosis, metastasis, and angiogenesis\(^{39-41}\). Alternatively, agents inactivating NF-\(\kappa\)B or STAT3 can inhibit cancer growth through undergoing apoptosis. Park et al\(^{42}\) reported that bee venom inhibited prostate cancer growth through induction of apoptosis via inactivation of NF-\(\kappa\)B, and Saydmohammed et al\(^{43}\) demonstrated that Curcumin suppresses constitutive activation of STAT-3 by up-regulating protein inhibitor of activated STAT-3 (PIAS-3) in ovarian and endometrial cancer cells. Unexpectedly, my data revealed that SVT didn't affect the activity of NF-\(\kappa\)B and STAT3 in colon cancer cells, HCT116, although it was confirmed that SVT inhibit the colon cancer growth through induction of apoptosis via enhancement of DR expression and subsequent caspase cascade events. Taken together, as the activated apoptosis related pathway doesn't reflect all of the characteristics of the classical apoptotic pathways from cytochrome C release to caspase cascade events relying on anti-cancer agents and cancer cell type\(^{10,11}\), present study revealed partially different findings including conversely increased G1 or G2/M related cyclins, unaffected NF-\(\kappa\)B and STAT3 activity, from the previous findings.
Although further study is needed to reconfirm the results in this study and to elucidate more concrete mechanism, consequently, These present data provide that SVT could be useful candidate compounds to enhance tumor growth inhibiting ability of chemotherapeutics through overcoming the resistance via enhancement of DR expression and the related apoptosis.

V. References


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