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원 저

Snake Venom from *Vipera Lebetina Turanica* Inhibits Tumor in a PC-3 Cell Xenograft Model and PC-3 Cell Growth *in Vitro*

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국문초록

Vipera Lebetina Turanica 사독의 PC-3 세포성장 억제

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목적 : 이 연구는 *Vipera lebetina turanica*의 蛇毒藥鍼液(Snake venom toxin, SVT)이 in vitro에서 NF-κB의 활성억제와 apoptosis 관련 단백질의 발현 조절을 통하여 세포자멸사(Apoptosis)를 유도하는지 *in vivo*에서 또한 전립선 암세포주인 PC-3 세포의 성장을 억제하는지 살펴보고자 하였다.

방법 : SVT를 처리한 후 PC-3의 성장억제를 관찰하기 위해 WST-1 assay, CCK-8 assay를 시행하였고, Apoptosis evaluation에는 DAPI, TUNEL staining assay를 시행하였으며, Apoptosis regulatory proteins의 변화 관찰에는 western blot analysis를 시행하였고, apoptosis와 연관된 NF-κB의 활성 변화를 관찰하기 위해 EMSA시행하였으며, SVT의 핵내이동을 관찰하기 위해 Immunofluorescence Staining, Confocal immunocytochemistry를 시행하였으며, 전립암세포의 종양형성에는 흉선을 제거한 쥐에 Tumorigenecity study를 시행하였다.

결과 : PC-3 세포에 SVT를 처리한후, 전립선암세포의 성장, Apoptosis의 유발, Apoptosis관련 단백질의 발현, NF-κB의 활성, SVT의 PC-3세포 핵내 이동여부 및 흉선제거 후 PC-3 세포를 이식한 쥐의 종양형성과정에 미치는 영향을 관찰하여 다음과 같은 결과를 얻었다.

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1. PC-3 세포에서 SVT를 처리한 후 세포성장이 억제되고, 세포자멸사가 유도되며, 조절인자인 p53, caspase-3, -9는 증가되었고, Bcl-2는 감소되었다.
2. PC-3 세포에서 SVT를 처리한 후 NF-κB의 활성이 유의하게 감소되었다.
3. DAPI로 염색된 상태에서 SVT가 PC-3 세포의 핵내로 이동되는 것이 관찰되었다.
4. 흉선 제거 후 전립선 암세포주를 이식한 쥐에서 SVT를 피내로 주입한 결과 전립선암의 크기와 무게가 유의하게 감소하였다.

결 론 : 이상의 결과는 SVT가 NF-κB의 활성 억제를 통하여 인간 전립선암세포주인 PC-3의 세포자멸사를 유발함으로써 증식억제 효과가 있음을 입증한 것이며, 이를 재확인한 생체 연구에서의 긍정적인 결과는 향후 SVT의 전립선암의 예방과 치료에 대한 효과적인 치료제 개발에 초석이 될 것으로 기대된다.

핵심단어 : 사독, 전립선암, 세포자멸사, 이종이식, PC-3, *Vipera lebetina turanica*, NF-κB, p53, caspase-3, caspase-9, Bcl-2, Bax

I . Introduction

Prostate cancer is the most common cancer as well as the second leading cause of tumor-related deaths in men of western countries[1]. One out of nine men over 65 years of age is frequently diagnosed with prostate tumor in the United States[1,2]. Current therapies for prostate tumor, such as surgery, radical prostatectomy and radiation therapy, are effective in many patients with locally advanced disease, but many of these patients eventually have recurrence[3]. Cytotoxic chemotherapies do not show any significant improvement in patient condition, either due to the high resistance of prostate tumor cells against chemotherapeutic agents, which is responsible for 28,000 deaths per year [1,4]. In the normal prostate, organ homeostasis is maintained by a dynamic balance between the rate of cell proliferation and the rate of programmed cell death (apoptosis)[5]. Failure to undergo apoptosis has been implicated in tumor development and resistance to tumor therapy. Promotion of apoptosis in prostate tumor cell may lead to the regression of tumor cells, and improved prognosis of refractory disease[6,7]. Thus any agents inducing apoptosis may be useful for chemotherapy against prostate tumor[8].

Recent several studies have reported that NF-κB is constitutively activated in human prostate cancer tissue, androgen-insensitive human prostate carcinoma cells, and prostate cancer xenografts[9,10]. Although, it was reported that activation of NF-κB by lysophosphatidic acid promotes survival of PC-3 cells[9], Syrovets et al reported that inhibition of NF-κB activity by acetyl-boswellic acids promotes apoptosis in androgen-independent PC-3 cells in vitro and in vivo[10]. Li and Sarkar also reported that inactivation of NF-κB by soy isoflavone genistein contributes to increased apoptosis of human PC-3 cancer cells[11]. Kim et al also previously found that apoptotic cell death of neuroblastoma by peroxisome proliferators 15-deoxy-delta[12,14]-prostaglandin J2 was accompanied with inactivation of NF-κB[12]. Therefore, agents capable of suppressing NF-κB pathway may be potentially useful in the prevention and management of prostate cancer growth and resistance via induction of apoptotic prostate cancer cell death.

Elucidating important molecular mechanisms of specific toxin-receptor and/or ionchannel complexes have been largely studied in drug discovery using natural toxins[13-15]. A snake venom toxin (SVT) from Vipera lebetina turanica, is a group of basic peptides, and important factor V activator composed of 236 amino acids with six disulfide bonds formed by twelve cysteins[16]. It was reported that long chain snake toxin possessing five

disulfide bonds has higher affinity to the nicotinic acetylcholine receptor, and reduced fifth disulfide bond lowers binding affinity to Acetylcholine receptor (AchR)[17]. Michalet et al.reported that cys192-193 residue of subunit of AchR was binding target of snake toxin, and the disulfide bond of snake toxin may be core or additional specific binding amino acid residues[18,19]. Park et al. recently also found that SVT from Vipera lebetina turanica inhibited NF-κB activation and target gene expression through the interaction with the signal molecules(p50 and IKKs) in the NF-κB pathway[20].

In this study, we conducted an in vitro and in vivo analysis to evaluate the prostate cancer cell response to SVT from Vipera lebetina turanica in order to determine the ability of this venom toxin as a therapeutic agent to suppress prostate cell growth and resistance by inducing apoptotic cell death, and determine possible mechanisms related with inactivation of NF-κB signals.

Ⅱ. Material and Method

1. Materials

Snake Venom(SVT) from Vipera lebetina turanica was purchased from Sigma Chemical Co. (Saint Louis, MI, USA).

2. Cell Culture

The PC-3 human prostate cancer cell was obtained from ATCC(American Type Culture Collection, Rockville, MD). Prostate cells were cultured in RPMI-1640 medium(Life Technologies Inc., Gaithersberg, MD) supplement with 10% fetal calf serum (FCS; Collaborative Biomedical Products, Bedford, MA) and antibiotics, penicillin/streptomycin (100 unit/ml, Bioproducts, Walkersville, MD) Cell cultures were then maintained at 37℃ in a humidified atmosphere of 5% CO_2

3. Cell Viability Assay

WST-1 assay

Cells were plated at a density of 1×10^5 cells per well in 96-well plate and then subconfluent cells were exposed to different doses(10-25μM) of SVT for 12, 24, 48, 72 hr. After treatment, Cell viability was measured by WST-1 assay(Dojin Laboratory, Kumamoto, Japan) according to the manufacturer's instructions. WST-1 solution was added to cells in 96-well plates, cells were incubated at 37.5℃ for 1hr, and the optical density of each well was read at 450nm.

CCK-8 assay

Cells were plated at a density of 1×10^4 cells/well in 96-well plates and then subconfluent cells were exposed to different doses(0, 0.25 - 2 μg/ml) of SVT from *Vipera lebetina turanica* (Sigma, St. Louis, MO) for 24, 48 or 72 hr. After treatment, cell viability was measured by Cell Counting Kit-8(CCK-8) system(Dojindo Laboratory, Kumamoto, Japan) according to the manufacturer's instructions. CCK-8 solution was added to cells in 96-well plates, cells were incubated at 37°C for 1 hr, and the optical density of each well was read at 450nm.

4. Apoptosis Evaluation

Apoptosis assays were performed using the 4,6-diamidino-2-phenylindole(DAPI) staining. PC-3 cells were cultured in the absence or presence of increasing concentrations of SVT, and apoptosis induction were evaluated after 24 hr. Apoptotic cells were determined by the morphological changes after DAPI staining under fluorescence microscopic observation(DAS microscope, 100 or 200x; Leica Microsystems, Inc., Deerfield, IL). For each determination, three separate 100-cell counts were scored. Apoptosis was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the

total number of cells counted.

Apoptosis was also evaluated by TUNEL staining assay. In short, cells were cultured on 8-chamber slides. After treatment with SVT (0.25~2μg) for 24hr, the cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1h at room temperature. TUNEL assays were performed by using the in situ Cell Death Detection Kit(Roche Diagonostics GmbH, Mannheim, Germany) according to manufacturer's instructions. Total number of cells in a given area was determined by using DAPI nuclear staining. The apoptotic index was determined as the number of TUNEL-positive stained cells divided by the total cell number counted 100.

5. Immunofluorescence Staining

PC-3 cells were plated in chambered tissue culture slides at a density of 5×10^4cells/well in RPMI. The cells were then cultured with SVT (0.25~2μg) or vehicle. Twenty-four hours later, the cells were washed once with PBS and fixed with 4% paraformaldehyde for 20min, membrane-permeabilized by exposure for 2min to 0.1% Triton X-100 in phosphate-buffered saline, and placed in blocking serum(5% bovine serum albumin in phosphate-buffered saline) at room temperature for 2hr. The cells were then exposed to primary rabbit polyclonal antibody for active caspase-3(1 : 50 dilution) overnight at 4℃, After washes with ice-cold PBS followed by treatment with an anti-rabbit secondary antibody Alexa Fluor 568(Molecular Probes Inc., Eugene, OR, USA), 1 : 100 dilution, for 2hr at room temperature, immunofluorescence images were acquired using a confocal laser scanning microscope(TCS SP2, Leica Microsystems AG, Wetzlar, Germany) equipped with a 630×oil immersion objective.

6. Confocal Immunocytochemistry

To determine whether SVT could be uptaken into the cells, cells(1×10^5cells/cm^2) were cultured on the chamber slide(Lab-Tak II chamber slider system, Nalge Nunc Int., Napervill, IL, USA) and then treated by SVT labeled with superior Alexa Fluor 488 dye(Molecular Probe, Eugene, Oregon, USA). Cells were incubated for 24 hr at 37℃, and the cells were then fixed in 4% paraformaldehyde, membrane permeabilized by exposure for 5 min to 0.2% Triton X-100 in phosphate-buffered saline, and were placed in blocking serum(5% horse or goat serum in phosphate-buffered saline). Immunofluorescence images were acquired using a confocal laser scanning microscope(dual wavelength scan, MRC1024, Bio-Red, Hercules, CA, USA) with a 60 X oil immersion objective.

7. Western Blot Analysis

Cells were homogenized with lysis buffer[50 mM Tris pH 8.0, 150mM NaCl, 0.02% sodium azide, 0.2% SDS, 1mM PMFS, 10μl/ml aprotinin, 1% igapel 630(Sigma-Aldrich, St. Louis, MO, USA), 10mM NaF, 0.5mM EDTA, 0.1mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 23,000g for 1hr. Equal amount of proteins (80μg) were separated on a SDS/12%- polyacrylamide gel, and then transferred to a nitrocellulose membrane(Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were blocked for 2hr at room temperature with 5%(w/v) non-fat dried milk in Tris-buffered saline[10mM Tris(pH 8.0) and 150mM NaCl] solution containing 0.05% tween-20. The membrane was incubated for 5hr at room temperature with specific antibodies caspase-3, goat polyclonal p50 antibody(1 : 1000) (Santa Cruz, CA, USA Santa Cruz Biotechnology Inc.). The blot was then incubated with the corresponding conjugated anti- rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL western blotting detection system. The relative density of the protein bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software

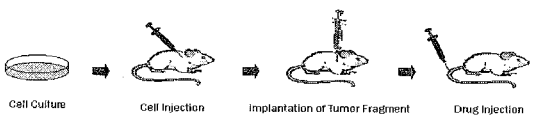
(UVP Inc., Upland, California).

8. Preparation of Nuclear Extracts and Electromobility Shift Assays

It was performed according to the manufacturer's recommendations(Promega, Madison, WI). Briefly, 1 × 10^6cells/ml was washed twice with 1 × PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000g for 1min, and the resulting supernatant was removed. Solution A (50mM HEPES, pH 7.4, 10mM KCl, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 0.1μg/ml phenylmethyl- sulfonyl fluoride, 1μg/ml pepstatin A, 1μg/ml leupeptin, 10μg/ml soybean trypsin inhibitor, 10μg/ml aprotinin, and 0.5% Nonidet P-40) was added to the pellet in a 2:1 ratio (v/v) and allowed to incubate on ice for 10min. Solution C(solution A + 10% glycerol and 400mM KCl) was added to the pellet in a 2:1 ratio (v/v) and vortexed on ice for 20min. The cells were centrifuged at 15,000g for 7min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [g^{-32}P] ATP for 10min at 37℃. Gel shift reactions were assembled and allowed to incubate at room temperature for 10 min followed by the addition of 1μl(50,000- 200,000cpm) of ^{32}P-labeled oligonucleotide and another 20min of incubation at room temperature. For supershift assays, nuclear extracts from cells treated with SVT (0.25 - 2μg/ml) were incubated with specific antibodies against the p50, p65 and Rel-A NF-κB isoforms for 1 hr before EMSA. For competition assays, nuclear extracts from cells treated with SVT (0.25 - 2μg/ml) were incubated with unlabelled NF-κB oligonuclaotide(50X, 100X and 200X) or labeled SP-1(100X) and AP-1(100X) for 30min before EMSA. Subsequently 1μl of gel loading buffer was added to each reaction and loaded onto a 6% nondenaturing gel and electrophoresed until the dye was three-fourths of the way down the gel. The gel was dried at 80℃ for 1hr and exposed to film overnight at 70℃. The relative density of the DNA-protein binding bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, California).

9. Tumorigenecity Studies in Athymic Nude Mice

For present study, we employed PC-3 human prostate cancer cells because they develop rapid tumors with high secretion of prostate-specific antigen(PSA) in the host. A total of 1 × 10^6 cells suspended in 50㎕ of media and 50㎕ of Matrigel (BD Biosciences, Bedford, MA) were inoculated s.c. into the right flank of sixteen 6-week-old mice by using a 27-gauge needle and divided into control and SVT groups which were 0.2mg/kg(5μg/100㎕ saline/25g mouse) of SVT group(SVT 0.2) and 0.4mg/kg(10μg/100㎕ saline/25g mouse) of SVT group(SVT 0.4). Whereas SVT group of animals received an i.d. injection of 5 μg/100㎕ or 10μg/100㎕ of saline every three days, the second group received an i.p. injection of 100㎕ saline alone and served as the control group. Every week, tumor growth was estimated in terms of volume of tumors(㎣) as a function of time (days).



10. Statistical Analysis

Data were analyzed using one-way analysis of variance followed by Tuckey test as a post hoc test. Differences were considered significant at $p<0.05$.

Ⅲ. Results

1. Inhibition of PC-3 Cell Growth

Morphological alteration of the cells was demonstrated in Fig. 1. Once the cells were exposed to SVT, the cells were not grown, and died in a dose dependent manner. To evaluate an effect of SVT on the cell growth of PC-3 cells, we analyzed cell viability using WST-1 assay and direct cell counting. SVT inhibited prostate cancer cell growth in a dose(0-2μg) dependent manner, the percentage of control significantly decreased by 1 and 2μg of SVT was 62.3±5.4 and 43.8±6.2 %(Fig. 2).

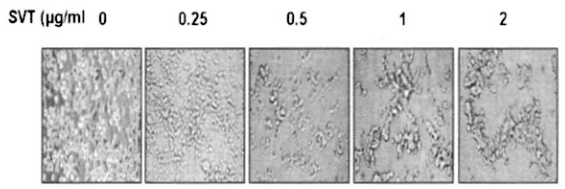


Fig. 1. Morphological changes of PC-3 cells by SVT
Morphological changes were observed under microscope(magnification, 200×). The figures are representative of three experiments, with triplicate of each experiment.

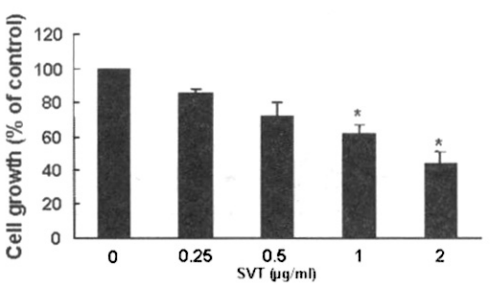


Fig. 2. Cell growth of PC-3 cells by SVT
Cell viability was determined by WST-1 assay as described under Method.

2. Induction of Apoptosis

To delineate whether the inhibition of cell growth by the SVT was due to increase of the induction of apoptosis, we evaluated change of the chromatin morphology of human prostate cancer cells using DAPI staining. Consistent with the loss of viability, apoptosis determined after 24hr treatment was increased in a dose dependent manner. The percentages of the control significantly increased by SVT 0.25, 0.5, 1 and 2 μg was 25.9±5.6, 54.9±7.9, 82.2±9.8 and 98.5±2.8% respectively(Fig. 3). We also evaluated PC-3 cell apoptosis by TUNEL assay. As shown in Fig. 3 TUNEL-positive cells (stained green) were dose-dependently increased in SVT treated PC-3 cells, and the nuclei(stained blue) were found to be condensed(Fig. 4).

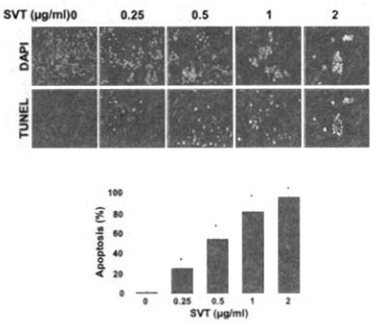


Fig. 3. Effect of SVT on induction of apoptosis of PC-3 cells
The apoptotic cells were examined by fluorescence microscopy after DAPI staining.

3. Expression of Apoptosis Regulatory Proteins

Execution of apoptosis occurs through activation of Bax and the caspase proteases and inactivation of Bcl-2. Caspases are a family of proteases that are expressed as inactive pro-enzymes in normal cells, and upon activation, they are capable of cleaving structural and functional proteins involved in key cellular processes[21]. The increase of apoptotic action was confirmed by the ability of SVT to induce caspase-3 and 9 activations. Fig. 4 reveals a western blot analysis of Bax, Bcl-2, caspase-3 and caspase-9 expressions in PC-3 cells treatment with a different dose of SVT. Compared with control, expression of the tumor

suppressor gene, p53, and active form of caspase-3 and 9 were increased by 0.25, 0.5, 1 and 2μg of SVT in a dose dependent manner, of which p53 significantly increased by 0.25, 0.5, 1 and 2μg of SVT was 207.8±7.2, 241.7±10.1, 270.7± 12.9 and 446.3±35.7%, and caspase-3 significantly increased by 0.25, 0.5, 1 and 2μg of SVT was 723±28.6, 1201±69.5, 1966±34.9 and 5155±415.5%, and caspase-9 significantly increased by 0.25, 0.5, 1 and 2μg of SVT was 1168±108.5, 1603±102.3, 1587±53.9 and 2146±154.4%, but Bcl-2 was decreased in a dose-dependent manner in the cells treated by SVT for 24hr(Fig. 4), which significantly decreased by 1 and 2μg of SVT was 6.5± 0.8 and 4.3±0.6 %.

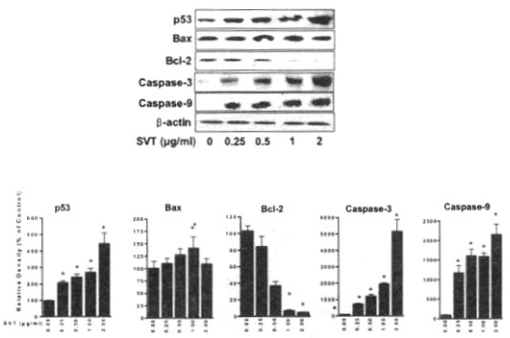


Fig. 4. Effect of SVT on expression of apoptosis related proteins in PC-3 cells

Equal amounts (50μg) of whole cell lysates were subjected to electrophoresis and analyzed by apoptosis regulatory molecules(p53, Bax, Bcl-2, caspase-3, and caspase-9). The relative density was analyzed by densitometry. Similar patterns of protein expression were obtained from three experiments. Values are mean ± S.D. of two experiments, with triplicate of each experiment.

4. Inhibition of NF-κB.

It was demonstrated that SVT negatively regulates nuclear transcription factor NF-κB in Astrocytes and RAW264.7 cells(Fig. 5). In addition, NF-κB is known to inhibitory transcription factor of apoptosis. To investigate the hypothesis whether SVT can inactivate NF-κB, and thereby prevent anti-apoptotic ability of NF-κB causing PC-3 cells go apoptosis, we assessed NF-κB activity in the cells treated for different concentration with for 24 hr SVT by EMSA. NF-κB was highly activated in this cell, however the activation of NF-κB was gradually decreased by the culture in the presence of SVT in the cell. The density of control significantly decreased by SVT 0.5, 1 and 2μg was 54.5±5.9, 56.5±7.8 and 53.5±2.8 in the cell(Fig. 6).

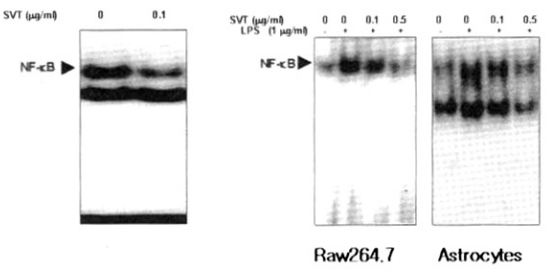


Fig. 5. Inhibition of NF-κB activity in RAW264.7 cells and Astrocytes by SVT

Activation of NF-κB was determined by electrophoretic mobility shift assay (EMSA), as described in Materials and Methods. Nuclear extracts from RAW264.7cells and Astrocytes treated either with 1 μg of LPS alone or with SVT(0.1, 0.5μg) were incubated in binding reactions of ^{32}P-labeled oligonucleotide containing the B sequence. NF-κB DNA binding activity was determined by EMSA.

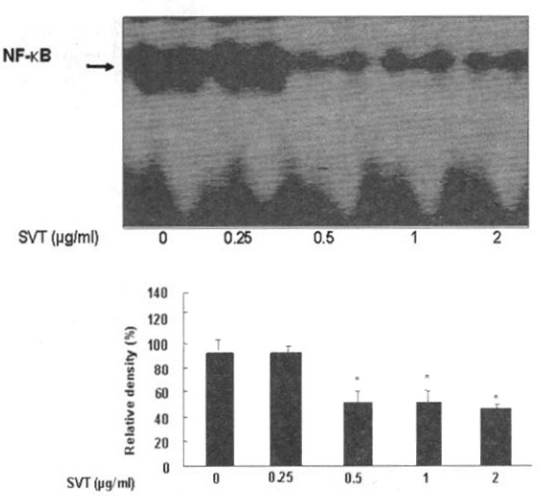


Fig. 6. Inhibition of NF-κB activity in PC-3 cells by SVT

Activation of NF-κB was determined by electrophoretic mobility shift assay(EMSA), as described in Materials and Methods. Nuclear extracts from PC-3 cells treated either with 1μg of LPS alone or with SVT(0.25, 0.5, 1 and 2μg) were incubated in binding reactions of ^{32}P-labeled oligonucleotide containing the B sequence. NF-κB DNA binding activity was determined by EMSA.

5. Uptake of SVT into Nucleus

It was demonstrated that SVT could be uptaken into nucleus of Astrocytes and RAW264.7 cells and have little cytotoxic effect on the cells(Fig. 7-9).

To investigate whether SVT can be uptaken into nucleus, and thereby inactivation of NF-κB and apoptotic cell death regulatory gene expression, we determined the location of SVT after treatment of cells with fluorescent dye labeled SVT. The uptake of the labeled SVT into the cells was shown under a confocal laser scanning microscope. As seen the Fig. 10, SVT was uptaken into the membrane and nucleus of cells. The translocation into the nucleus was evidenced by the merging of PI staining of nucleus and labeled SVT.

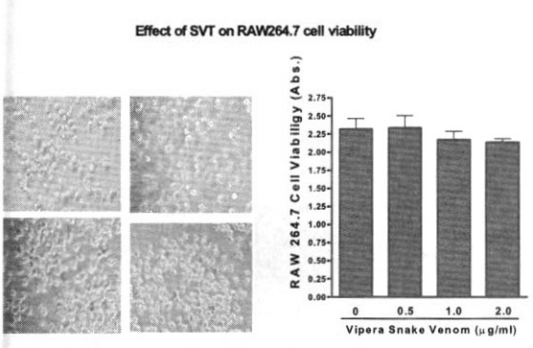


Fig. 7. Morphological changes and cell viability of RAW264.7 cells by SVT
Cell viability was determined by CCK-8 assay. Values are mean ± S.D. of three experiments, with triplicate of each experiment. Cell morphological changes were observed under microscope (magnification, 200x).

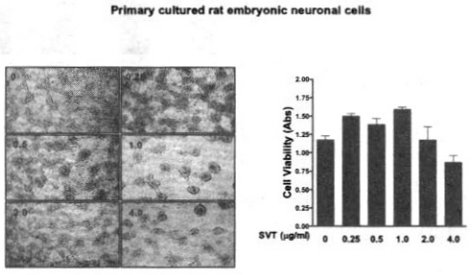


Fig. 8. Morphological changes and cell viability of primary cultured Rat embryonic neuronal cells by SVT
Cell viability was determined by CCK-8 assay. Values are mean ± S.D. of three experiments, with triplicate of each experiment. Cell morphological changes were observed under microscope (magnification, 200x).

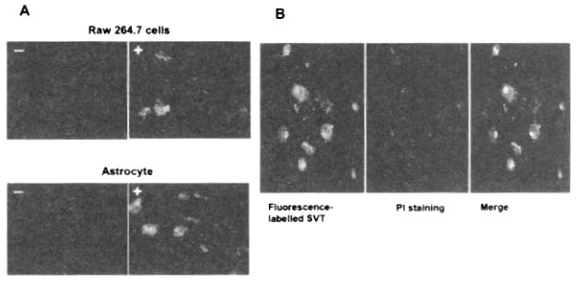


Fig. 9. Uptake of SVT into Astrocytes and RAW264.7 cells
RAW264.7 cells and Astrocytes were treated with Alexa Fluor 488 dye-labeled SVT for 24 hr. Cells were washed, and the uptaken of labeled SVT was shown by a confocal scanning microscope (magnification, 630x). Double staining (Merge) with fluorescence labeled SVT and DAPI staining demonstrating the localization of SVT in the nucleus. Each figure is representative of three similar experiments.

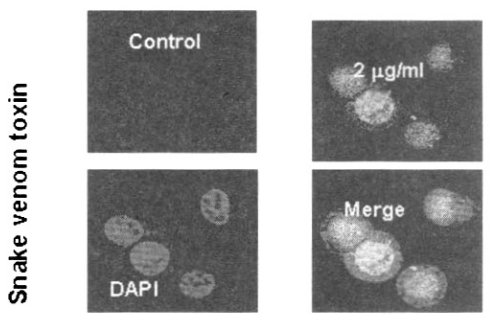


Fig. 10. Uptake of SVT into PC-3 cells
PC-3 cells were treated with Alexa Fluor 488 dye-labeled SVT for 24 hr. Cells were washed, and the uptaken of labeled SVT was shown by a confocal scanning microscope(magnification, 630x). Double staining(Merge) with fluorescence labeled SVT and DAPI staining demonstrating the localization of SVT in the nucleus. Each figure is representative of three similar experiments.

6. Effect of SVT on Tumorigenecity of PC-3 cells in an Athymic Nude Mouse model.

Because SVT was observed to be effective in

inhibiting the growth of PC-3 cells *in vitro*, we next investigated whether these results could be translated into an *in vivo* xenograft model. SVT did not cause any loss in the body weight, food intake, or exhibited apparent signs of toxicity in animals. Implantation of PC-3 cells onto nude mice produced visible tumors in mice with a mean latent period of 18 days. The average volume and weight of tumors in control mice increased as a function of time and reached a preset end point of 0.87± 0.1㎖ and 1.3 ± 0.2g in 30days after inoculation. However, at this time, the average tumor volume and weight of SVT 0.2 and SVT 0.4 was decreased in a dose dependent manner, which was 0.82 ± 0.2㎖ and 1 ± 0.2g, and 0.6 ± 0.2㎖ and 0.8 ± 0.2g respectively(Fig. 11). We also evaluated whether or not i.d .Injection of SVT into mice leads to inhibition of PC-3 cell growth in nude mice. As i.p. Injection of saline was responsible for poor growth of PC-3 cells in the control, we adopted SVT 0.2 as the alternative of the control. Compared with SVT 0.2, Tumor growth inhibition ratio of SVT 0.4 was 50.41% in 30days after inoculation(Fig. 12-15).

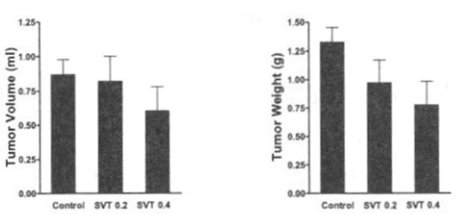


Fig. 11. Effect of SVT on volume and weight of tumor in a PC-3 cell xenograft nude mice

A total of 1 × 106cells suspended in 50㎕ of media and 50㎕ of Matrigel (BD Biosciences, Bedford, MA) were inoculated s.c. into the right flank of sixteen 6-week-old mice by using a 27-gauge needle and divided into control and SVT groups which were 0.2mg/kg (5㎍/100㎕ saline/25 g mouse) of SVT group(SVT 0.2) and 0.4mg/kg (10㎍/100㎕ saline/25g mouse) of SVT group(SVT 0.4). Whereas SVT group of animals received an i.d. injection of 5㎍/100㎕ or 10㎍/100㎕ of saline every three days, the second group received an i.p. injection of 100㎕ saline alone and served as the control group. Every week, tumor growth was estimated in terms of volume and weight of tumors (㎣ and g) as a function of time

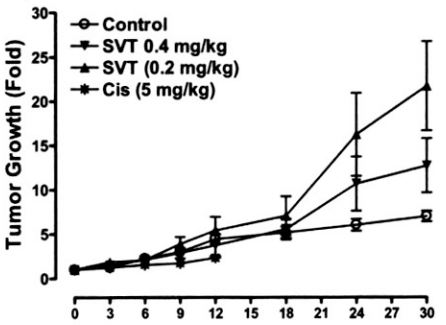


Fig. 12. Inhibition of tumor growth by SVT in a PC-3 cell xenograft nude mice
Implantation of PC-3 cells onto nude mice produced visible tumors in mice with a mean latent period of 18 days. As i.p. Injection of saline was responsible for poor growth of PC-3 cells in the control, we adopted SVT 0.2 as the alternative of the control. Values are mean ±S.D. of two experiments, with triplicate of each experiment.

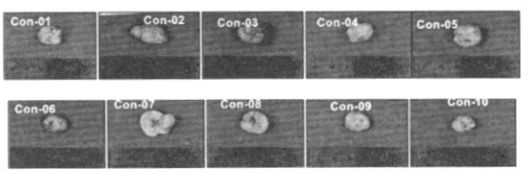


Fig. 13. Tumor mass extracted from the Control
These are photographs of tumor mass taken out of 10 PC-3 cells xenograft nude mice in the control group after sacrifice.

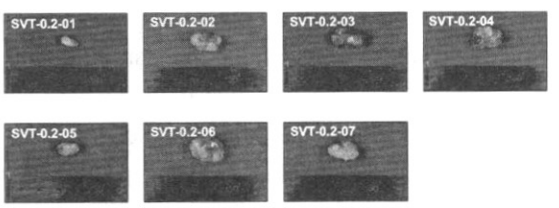


Fig. 14. Tumor mass extracted from SVT 0.2
These are photographs of tumor mass taken out of 7 PC-3 cells xenograft nude mice treated with low dose(0.2 mg/Kg) of SVT in the SVT 0.2 group after sacrifice.

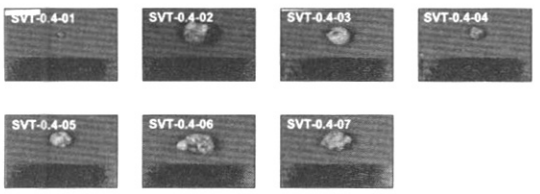


Fig. 15. Tumor mass extracted from SVT 0.4
These are photographs of tumor mass taken out of 7 PC-3 cells xenograft nude mice treated with high dose(0.4 mg/Kg) of SVT in the SVT 0.4 group after sacrifice.

Ⅳ. Discussion

The central and novel finding in the present study is the identification of *in vitro* and *in vivo* anticancer efficacy of SVT from *Vipera lebetina turanica* against advanced human prostate carcinoma PC-3 cells. Most of the present available cytotoxic anticancer drugs mediate their effect via induction of apoptosis in cancer cells[22,23], and induction of apoptosis is suggested as one of the major mechanisms for the targeted therapy of various cancers including prostate cancer[22-25]. In case of advanced prostate cancer, cancer cells become resistant to apoptosis and do not respond to cytotoxic chemotherapeutic agents[24]. Therefore, the agents that induce apoptotic cell death of prostate cancer cells could be useful in controlling this malignancy[25]. Consistent with this approach, our data showing an induction of apoptotic cell death in prostate cancer control suggest that extremely low concentration (below 2μg/ml) of natural toxin (SVT) could be useful as a anti-cancer agent.

It has been well established that NF-κB is an important element in regulating cell growth or apoptosis of tumor cells, including prostate cancer cells[26]. Down regulated NF-κB signals by SVT is consistent with cell growth inhibition. These data suggest that NF-κB signal may be significant contributor in SVT-induced PC-3 cell death. Recently several studies have reported that NF-κB is constitutively activated in human prostate cancer tissue, androgen-insensitive human prostate carcinoma cells, and prostate cancer xenografts[9,10]. Moreover, compounds inhibiting NF-κB have shown to induce apoptotic cell death of PC-3 cells[10,11]. Kim et al also previously found that apoptotic cell death of neuroblastoma by peroxisome proliferators 15-deoxy-delta 12, 14-prostaglandin J2 was accompanied with inactivation of NF-κB[12]. Therefore, agents capable of suppressing NF-κB pathway may be potentially useful in the prevention and management of prostate cancer growth via induction of apoptotic prostate cancer cell death. At the molecular level, SVT inhibits constitutively activated NF-κB signaling by impairing IκBα phosphorylation with inhibition of p50 translocation. Consistent with the present finding, we previously found that SVT binds with NF-κB, IKKα and IKKβ resulting in down regulation of NF-κB activity in Raw 264.7 cells and Astrocytes[20]. Moreover, reduced SVT-induced apoptotic cell death was found in the cells transfected with mutant p50, IKKα and β in which the cysteine residue was replaced with other amino acids. These data therefore suggest that SVT-induced PC-3 cell death could be involved in the blocking NF-κB activation, and this effect may be due to direct binding of SVT to NF-κB signal molecules having sulfhydryl group of their active cites. Direct inhibition of DNA binding activity of NF-κB in the nucleus is also possible since we found that SVT translocated into nucleus in which p50 or p65 could bind to IκB binding elements of target genes. Consequently, the reduced nuclear translocation of NF-κB proteins were associated with down-regulation of the constitutively overexpressed or NF-κB-dependent anti-apoptotic proteins.

Several genes (proteins) such as Bcl-2, and/or up-regulated apoptotic genes: Bax, caspase-3 and -9, and p53 are regulated by NF-κB. Bcl-2 protein is prototypic cell death regulator whose function is modulated by complex homo- and

heterodimerizations with their proapoptotic homologues such as Bax[27,28]. Bcl-2 delays or prevents apoptotic cell death to virtually all of the chemotherapeutic agents, suggesting that it may be crucial for the antitumor activity[28-30]. Deregulated overexpression of Bcl-2 has been described in numerous malignant tissues including prostate cancer[27,28]. In addition, antisense Bcl-2 oligonucleotide constructs or down-regulation of Bcl-2 can be utilized to sensitize apoptosis-resistant prostate cancer cells to chemotherapeutic agents[29]. This altered expression of Bcl-2 family members triggered the activation of initiator caspase-9 and -8 followed by activation of effector caspase-3. Activation of caspase-3 activity has been known to significant in the cancer cell death, and compounds inhibiting caspase-3 activity have been suggested as powerful agents for cancer therapy. For these reasons, Bcl-2[28,30,31] and caspase-3[32-34] have been considered as a major new strategic target for the development of gene knockdown therapies, and activation of caspase-3 may be significant in the prostate epithelial cancer cell death[34-36]. In fact, in our result showed that caspase-3 was significantly activated by SVT. These data suggest that inhibition of NF-κB and activation of caspase-3 could be critical in the SVT-induced PC-3 cell death.

The possibility of usage of natural toxins as pharmaceutical applications have been demonstrated with several toxins including SVT in various *in vitro* or animal model as well as clinical studies[20,37-39]. Divergency in biological activities of various proteins isolated from snake venom has been reported. Siigur et al. demonstrated fibrolytic enzymes from Viper lebetina snake venom[40]. Saxatilin isolated from Korean snake(Gloydius saxatilis) has been shown to inhibitory effect on platelet aggregation, human umbilical vein endothelial cell proliferation and smooth muscle cell migration[39]. Salmosin, a disintegrin purified from a Korean snake (Agkistrodon halys brevicaudus) venom, interacts with integrin alpha(v)beta(3),and induce apoptotic cell death by competing with the extracellular matrix(ECM) for direct binding to integrin alpha(v)beta(3) on the cell surface. Out of several *in vitro* and/ or *in vivo* anti-cancer studies employing snake venom toxin, Swenson et al. demonstrated that Contortrostatin(CN) isolated from venom of the Southern Copperhead snake has anti-invasive and anti-adhesive activity on tumor cells and endothelial cells *in vitro*, and binds to integrins alphavbeta3, alphavbeta5, and/or alpha5beta1, and that *In vivo* studies using the human metastatic breast cancer cell line MDA- MB-435, in an orthotopic xenograft model in nude mice, CN has potent anti-tumor and anti-angiogenic activity, and he have also revealed that i.v. delivery of liposomal delivery(LCN) leads to potent antiangiogenic activity in the orthotopic, xenograft human mammary tumor model. Markland et al. found that In a xenograft nude mouse model with intraperitoneal introduction of OVCAR-5 cells, CN not only significantly inhibited ovarian cancer dissemination in the nude mouse model, but it also dramatically prevented the recruitment of blood vessels to tumors at secondary sites. our present date suggested that a promising result of tumor growth inhibition in a human prostate cancer PC-3 cell xenograft nude mice by I.d. injection of SVT reconfirmed the *in vitro* effects.

Thus, our present findings showing the *in vitro* and *in vivo* anticancer efficacy of SVT, with mechanistic rationale including apoptosis induction, against advanced human prostate cancer cells and preclinical human prostate cancer models could confirm a basis for the development of SVT as a novel agent for human prostate cancer prevention and/or intervention without safety apprehension.

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