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Scorpion venom component III inhibits cell proliferation by modulating NF-κB activation in human leukemia cells

XIANGFENG SONG[1,2], GUOJUN ZHANG[2], AIPING SUN[2], JIQIANG GUO[2], ZHONGWEI TIAN[3], HUI WANG[2] and YUFENG LIU[1]

[1]Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou; [2]Department of Immunology, Xinxiang Medical University, Xinxiang; [3]Department of Dermatology, The First Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, P.R. China

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Abstract. Scorpion venom contains various groups of compounds that exhibit anticancer activity against a variety of malignancies through a poorly understood mechanism. While the aberrant activation of nuclear factor κB (NF-κB) has been linked with hematopoietic malignancies, we hypothesized that scorpion venom mediates its effects by modulating the NF-κB signaling pathway. In the present study, we examined the effects of scorpion venom component III (SVCIII) on the human leukemia cell lines THP-1 and Jurkat and focused on the NF-κB signaling pathway. Our results showed that SVCIII inhibited cell proliferation, caused cell cycle arrest at G1 phase and inhibited the expression of cell cycle regulatory protein cyclin D1 in a dose-dependent manner in THP-1 and Jurkat cells. SVCIII also suppressed the constitutive NF-κB activation through inhibition of the phosphorylation and degradation of IκBα. NF-κB luciferase reporter activity was also inhibited by SVCIII. Our data suggest that SVCIII, a natural compound, may exert its antiproliferative effects by inhibiting the activation of NF-κB and, thus, has potential use in the treatment of hematopoietic malignancies, alone or in combination with other agents.

Introduction

Scorpion venom contains various groups of compounds that exhibit a wide range of biological properties and actions in cells. The general composition and expression level of scorpion venom depends on genetic variation and geographical environment (1,2). The scorpion *Buthus martensii* Karsch (BmK) and its products have been used as a traditional Chinese medicine for thousands of years. Traditional healers use scorpions to treat various types of condition, such as epilepsy, rheumatism and cancer. It has previously been reported that crude scorpion venom or isolated peptides from scorpion venom may inhibit the proliferation of cancer cells and induce cell apoptosis (3,4). However, the antitumor molecular mechanisms are poorly understood.

Nuclear factor κB (NF-κB) is an important transcription factor, which plays a part in many cellular activities such as proliferation and activation of immunocytes, development of T and B lymphocytes and cell apoptosis (5). However, substantial evidence also indicates that NF-κB plays a pivotal role in the onset and development of malignancies. Recent observations have shown that there is a close relationship between NF-κB and hematopoietic malignancies such as leukemia, lymphoma and multiple myeloma (6,7), as aberrant activation of the NF-κB pathway is involved in the pathogenesis of these diseases. Moreover, some studies have suggested that blocking the NF-κB signaling pathway can cause tumor cells to cease proliferation, die, or become more sensitive to the action of antitumor agents (7). The NF-κB signaling pathway has therefore become a promising target for cancer therapy. In the present study, we attempted to elucidate the antiproliferation and cell cycle arresting properties of scorpion venom component III (SVCIII) from BmK venom and its effects on the NF-κB signaling pathway in human leukemic cell line Jurkat and THP-1 cells.

Materials and methods

Chemicals. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (Carlsbad, CA, USA). TransFast™ Transfection Reagent was obtained from Promega Corporation (Madison, WI, USA). NF-κB luciferase reporter plasmid was a gift from Dr Luan Haojiang (US National Institutes of Health). Antibodies to cyclin D1, IκBα and p-IκBα were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Nuclear extract kit was purchased from Active Motif (Carlsbad, CA, USA). Chemiluminescent electrophoretic

Correspondence to: Professor Hui Wang, Department of Immunology, Xinxiang Medical University, Xinxiang, Henan 453003, P.R. China
E-mail: wanghui@xxmu.edu.cn

Professor Yufeng Liu, Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, Henan 450052, P.R. China
E-mail: lyf6012@tom.com

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mobility shift assay (EMSA) kit was purchased from Beyotime Institute of Biotechnology (Nantong, China). All other reagents used in the study were of analytical grade and purchased locally.

Scorpion venom. BmK venom was extracted by mild electrical stimulation of the telsons and dissolved in 0.02 M phosphate buffer, pH 7.2, and centrifuged at 10,000 x g for 15 min at 4˚C. Gel chromatography was utilized to isolate partial peptide fractions from crude scorpion venom. Seven fractions were obtained and named scorpion venom components (SVC)I, II, III, IV, V, VI, VII, respectively. The molecular weight of SVCIII was calculated to be approximately 70-80 kDa through comparison with protein markers of known molecular weights run in a 12% SDS-PAGE.

Cell culture and treatments. The THP-1 (human acute monocytic leukemia) cell line was provided by the Southern Medical University, and the Jurkat (human T lymphoma) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in 50-ml plastic flasks in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin and incubated in a 5% CO_2 humidity incubator at 37˚C. The medium was refreshed three times a week. Cells in log phase were seeded in sterile 6-, 24- or 96-well plates with a fixed number in each well and then treated with varying amounts of SVCIII for 48 h.

Cell viability assay by MTT. Cell viability was determined by MTT assay. Cells were seeded in a 96-well plate at a density of $1x10^5$ cells per well and treated with various concentrations (0, 1, 5, 10, 20, 30, 40 and 50 μg/ml) of SVCIII for 48 h. MTT dye was added to each well for the last 4 h of treatment. When purple precipitates were visible, the medium was carefully discarded. The formazan crystals were dissolved by adding 200 μl of dimethyl sulfoxide to each well. The cell viability index was calculated by measuring the absorbance value at 570 nm.

Flow cytometry for cell cycle analysis. A cell cycle assay was performed using propidium iodide (PI) staining of the nuclei. Following treatment for 48 h with SVCIII, cells were fixed in 70% cold alcohol overnight and then centrifuged. The pellet was re-suspended in 500 μl PI staining buffer (250 μg/ml PI, 10 μg/ml RNase in PBS) in a dark room for 30 min at room temperature and analyzed with a flow cytometer. For each measurement, at least 10,000 cells were counted.

NF-κB luciferase reporter luciferase assay. To determine the effect of SVCIII on NF-κB activation, cells were transiently transfected with a NF-κB luciferase reporter plasmid. Cells were seeded in 24-well plates (10^5/well) and transfected with 0.5 μg of a NF-κB luciferase reporter plasmid or pGL3 basic as a negative control using TransFast™ Transfection Reagent according to the manufacturer's instructions and co-transfected with 40 ng of pRL-TK *Renilla* luciferase vector to control transfection efficiency. Transfected cells were exposed to SVCIII for 6 h. Cells were then harvested and lysed according to the manufacturer's instructions. Supernatants were analyzed for firefly and *Renilla* luciferase activity using the dual-luciferase reporter assay system.

EMSA. To assess NF-κB activation, EMSA was performed according to the manufacturer's instructions for the Chemiluminescent EMSA Kit. Biotin-labeled double-stranded oligonucleotides were used which included commercially available consensus NF-κB gel shift oligonucleotide 5'-biotin-AGTTGAGGGGACTTTCCCAGG-3'. Specific binding was confirmed by competition experiments with a 100-fold excess of unlabeled or mutated oligonucleotides. The bands were detected by enhanced chemiluminescent (ECL) assay kit.

Cell extracts and western blotting. Nuclear extracts were isolated using a nuclear extract kit. Cells were briefly washed twice with ice-cold PBS/phosphatase inhibitors and incubated in 500 μl of hypotonic buffer for 15 min on ice. Subsequently, 25 μl detergent was added and the cells were vortexed at the highest setting and centrifuge suspended for 30 sec at 14,000 x g at 4˚C. Nuclei were washed with 50 μl complete lysis buffer and vortexed for 10 sec at the highest setting. Thereafter the lysate was incubated for 30 min on ice and centrifuged for 10 min at 14,000 x g. Protein concentrations were determined using the Bradford assay. Proteins were resolved by 12% SDS-PAGE gels, transferred onto a PVDF membrane and subjected to western blot analysis using anti-cyclin D1, IκBα and p-IκBα antibody. Proteins were visualized with an ECL assay kit according to the manufacturer's instructions.

Statistical analysis. Data are presented as mean ± S.D. and one-way analysis of variance was used to identify significant differences among the results. Statistical significance was defined as $P<0.05$.

Results

Effect of SVCIII on cell viability. MTT assay was used to determine the effect of SVCIII on cell viability. As shown in Fig. 1, the cell viability of THP-1 and Jurkat cells was decreased by SVCIII in a dose-dependent manner. The percentage of viable THP-1 cells following treatment with 1, 5, 10, 20, 30, 40 and 50 μg/ml of SVCIII decreased to 83.4, 74.7, 65.3, 55.0, 46.9, 38.2 and 33.4% respectively. Viability of Jurkat cells after exposure to increasing concentrations of SVCIII was reduced to 91.9, 86.7, 77.3, 68.8, 58.2, 49.4 and 41.3% respectively (Fig. 1). However, there were no significant differences in the human peripheral blood lymphocytes (PBLs) between SVCIII-treated cells and controls. The IC_{50} value was calculated to be 29 μg/ml for THP-1 and 39.6 μg/ml for Jurkat. The concentrations 1/2 IC_{50} and IC_{50} were used to investigate the effects of venom in subsequent experiments.

Effect of SVCIII on cell cycle distribution. We then tested the effect of SVCIII on cell cycle distribution using flow cytometry. A dose-dependent increase in the G1 phase cell population was observed from 45.1% in controls to 65.1 and 74.9%, respectively, due to SVCIII treatment at 1/2 IC_{50} (15 μg/ml) and IC_{50} (30 μg/ml) concentration for THP-1, and from 44.4% in controls to 56.4 and 65.7%, respectively, due to SVCIII treatment at 1/2 IC_{50} (20 μg/ml) and IC_{50} (40 μg/ml) concentration for Jurkat. A decrease in the S phase cell population was observed from 44.7% in controls to 25.6 and 17.3%, respectively, due to SVCIII treatment at 1/2 IC_{50} (15 μg/ml) and IC_{50} (30 μg/ml)

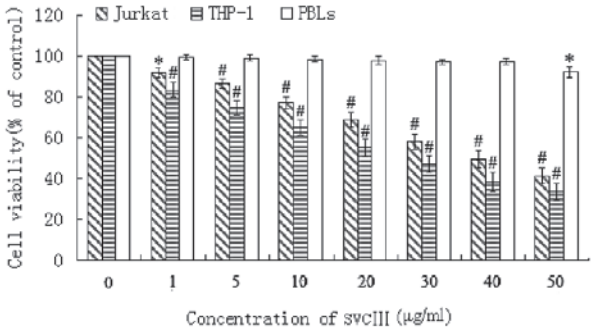

Figure 1. Effects of SVCIII on the viability of normal human lymphocytes (THP-1 and Jurkat cells). Cells were treated with various concentrations of SVCIII for 48 h. The index of cell viability was calculated by measuring the absorbance value at 570 nm. Data are expressed as percentages of the control value. *P<0.05 and #P<0.01, SVCIII-treated groups compared to the control (0 μg/ml).

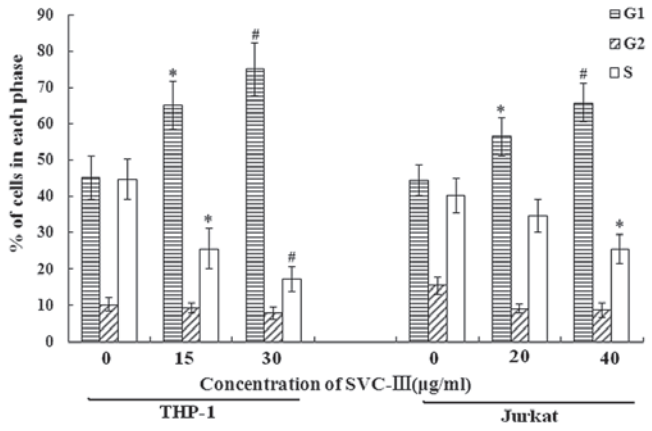

Figure 2. Effects of SVCIII on the cell cycle. THP-1 cells were treated with SVCIII at concentrations of 15 and 30 μg/ml while Jurkat cells were treated with SVCIII at concentrations of 20 and 40 μg/ml respectively for 48 h. A cell cycle assay was performed using propidium iodide staining of the nuclei by flow cytometry. Histograms show the percentages of cells at various phases of cell cycle. Each value is expressed as mean ± S.D. (n=3). *P<0.05 and #P<0.01, SVCIII-treated groups compared to the control (0 μg/ml).

concentration for THP-1, and from 40.2% in controls to 34.6 and 25.6%, respectively, due to SVCIII treatment at 1/2 IC_{50} (20 μg/ml) and IC_{50} (40 μg/ml) concentration for Jurkat (Fig. 2). These results indicate that SVCIII inhibits cell growth through arrest at G1 phase and reduces transition to the S and G2/M phases of the cell cycle in both THP-1 and Jurkat cells.

Effect of SVCIII on cyclin D1 protein. Cyclin D1, an NF-κB-regulated gene, is required for transition from G1 to S phase and plays a vital role in cell proliferation. We, therefore, examined whether SVCIII suppresses the expression of cyclin D1 protein. As shown in Fig. 3, SVCIII significantly inhibited the expression of cyclin D1 in a dose-dependent manner in both cell types. This result suggests a potential mechanism for how SVCIII suppresses tumor cell proliferation.

Effect of SVCIII on NF-κB activation. In order to determine whether NF-κB is involved in cell growth suppression induced

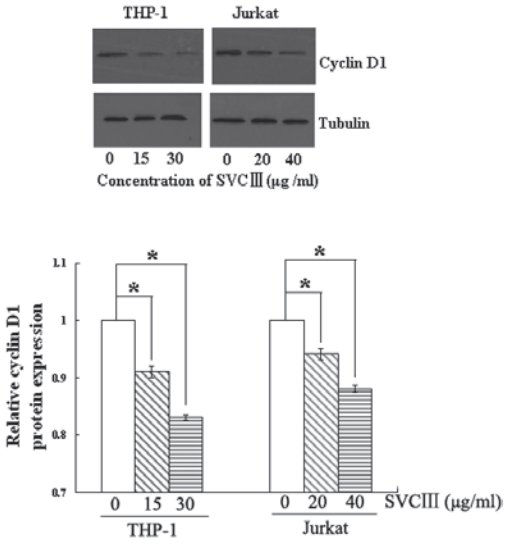

Figure 3. Effects of SVCIII on cyclin D1 protein. THP-1 cells were treated with SVCIII at concentrations of 15 and 30 μg/ml while Jurkat cells were treated with SVCIII at concentrations of 20 and 40 μg/ml for 24 h. Protein from the total cell lysate was subjected to SDS-PAGE and western blot analysis for cyclin D1 protein. Tubulin was used as an internal control. Representative results are shown from three independent experiments. *P<0.05, SVCIII-treated groups compared to the control (0 μg/ml).

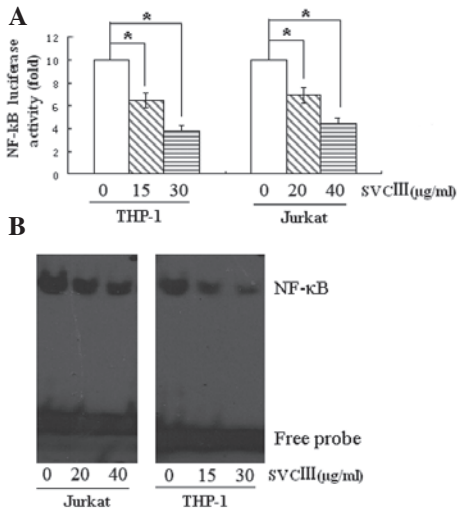

Figure 4. Effect of SVCIII on NF-κB activation. (A) Cells were transiently transfected with a NF-κB luciferase reporter plasmid or pGL3 empty vector and then were exposed to SVCIII (at concentrations of 15 and 30 μg/ml for THP-1 cells, 20 and 40 μg/ml for Jurkat cells) for 6 h. Cells were lysed and supernatants were analyzed for firefly and *Renilla* luciferase activity using the dual-luciferase reporter assay system. *P<0.01. (B) Cells were incubated with SVCIII for 6 h. Nuclear extracts were then prepared and assayed for NF-κB activation by electrophoretic mobility shift assay.

by SVCIII, we first measured NF-κB-luciferase activity by using a luciferase plasmid containing six tandem NF-κB sites as a minimal promoter. Fig. 4A showed that treatment with SVCIII resulted in a significant decrease in NF-κB-luciferase activity.

We then examined the NF-κB activation using EMSA. Exposure of cells to SVCIII led to a decrease in NF-κB-DNA binding in a dose-dependent manner (Fig. 4B). The suppression

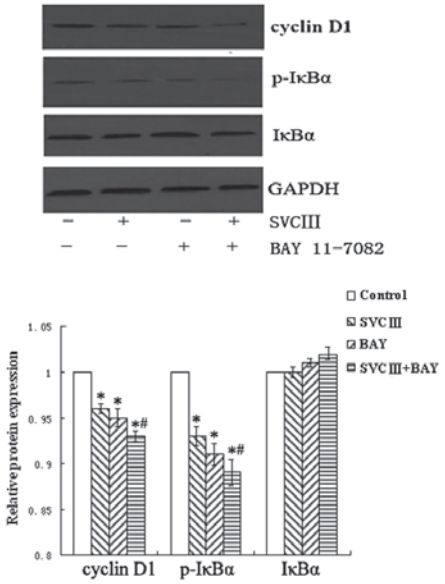

Figure 5. THP-1 cells were incubated with BAY11-7082 (1 μM) alone or in combination with SVCIII (30 μg/ml) for 24 h. Protein from the total cell lysate was subjected to SDS-PAGE and western blot analysis using anti-IκBα, p-IκBα, cyclin D1 and GAPDH antibodies. Representative results are shown from three independent experiments. *P<0.05, SVCIII-treated groups compared to the control; #P<0.05, SVCIII in combination with BAY11-7082 groups compared with SVCIII or BAY11-7082 alone.

of NF-κB-DNA binding activity was consistent with luciferase reporter activity. These results suggest that SVCIII inhibits NF-κB activation.

Effect of SVCIII on IκBα. The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα. To elucidate the signaling pathways involved in the suppression of NF-κB activation, we pretreated THP-1 cells with NF-κB inhibitor BAY11-7082 for 1 h and then subjected the cells to SVCIII. Although the expression of p-IκBα was markedly inhibited by SVCIII treatment, it was further decreased by the pre-treatment with the NF-κB inhibitor (Fig. 5). Next, we investigated whether the NF-κB inhibitor would also suppress the expression of cyclin D1. As expected, NF-κB inhibitor led to a decrease in the expression of cyclin D1, whereas it was further decreased by the NF-κB inhibitor combined with SVCIII (Fig. 5).

Discussion

In the present study, we investigated the effects of SVCIII on cell growth in the THP-1 and Jurkat cell lines, as well as on the NF-κB signaling pathway. We found that SVCIII inhibited the cell proliferation and cell cycle arrest at G1 phase in a dose-dependent manner, and suppressed NF-κB activation through inhibition of IκBα phosphorylation, degradation and p65 nuclear translocation.

It is well established that normal cells divide and create new cells only when needed. One of the hallmark characteristics of cancer cells is their uncontrolled proliferation (8). Our results demonstrated that SVCIII inhibited cell proliferation in human THP-1 and Jurkat cells in a dose-dependent manner. These results agree with previous reports that scorpion venom inhibited the growth of lymphoma (3,9), leukemia (10), neuroblastoma (4), gliomas (11-13), breast cancer (14) and prostate cancer (15,16).

It is well known that cell proliferation is closely related to cell cycle distribution. Under normal conditions, cells are believed to be in the G0 phase in most mammals. Cells progress through the cell cycle phase from G0/G1 to S after stimulation from extracellular signals. It was demonstrated that scorpion venom induced cell cycle arrest mainly in the G0/G1 phase and decreased in the S phase (3). The analysis of cell cycle distribution in the present study also showed that SVCIII inhibited cell proliferation with cell cycle arrest at the G1 phase and reduced transition to the S phase and G2/M phases of the cell cycle in a dose-dependent manner. This reinforces the evidence that suppression of cell cycle transition is involved in the SVCIII-induced antitumor action in human leukemia cells.

NF-κB plays a pivotal role in physiological immune reactions, as well as in the onset and maintenance of malignancies (17-20). It targets many genes that promote tumor progression, cell survival, proliferation, angiogenesis and metastasis (21-23). Aberrant or persistent activation of NF-κB is believed to be an important mechanism in the generation of various tumor types (24,25). In this study, we investigated the activity of NF-κB using the luciferase reporter gene and EMSA. Results showed that SVCIII inhibited the activity of NF-κB in a dose-dependent manner as well. This suggests that SVCIII prevents the binding of NF-κB to its target gene, and thus downregulates the expression of NF-κB-regulated gene products.

NF-κB is expressed in the cytoplasm of virtually all cell types. NF-κB activation is initiated by the signal-induced degradation of IκB proteins (26,27). In the classical NF-κB signaling pathway, IκB proteins are phosphorylated by an activated IκB kinase (IKK) complex and then degraded by the proteasome. The degradation of IκB allows NF-κB protein to translocate to the nucleus and bind to their cognate DNA binding sites to regulate the transcription of many genes (28,29). We found that the suppression of NF-κB activation was accompanied by inhibition of IκBα phosphorylation and degradation. Moreover, SVCIII also inhibited p65 nuclear translocation. Therefore, the inhibition of cell proliferation by SVCIII may be associated with downregulation of constitutive NF-κB activation.

It is clear that NF-κB transcription factor regulates expression of various genes, including cyclin D1 which has been linked with proliferation of tumor cells. Cyclin D1 modulates the cell cycle transition from G1 to S phase and is overexpressed in a variety of human malignancies (30,31). To reveal the inhibitory mechanism of SVCIII on cell proliferation, we investigated the effect of this compound on the cell cycle. It was found that treatment with SVCIII significantly inhibited the expression of cyclin D1 in a dose-dependent manner. These results suggest a molecular mechanism for the manner in which SVCIII suppresses tumor cell proliferation. Further studies are required to clarify the effects of SVCIII on other signaling pathways.

In conclusion, this study has demonstrated that SVCIII suppresses cell proliferation and cell cycle arrest at the

G1 phase by targeting the NF-κB signal pathway in THP-1 and Jurkat cells. This suggests that SVCIII may have a potential and/or adjuvant therapeutic application in the treatment of human leukemia.

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