

ORIGINAL ARTICLE

Effect of spider venom on inhibition proliferation of TE13 cells *in vivo* and *in vitro*

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Abstract

Background: The aim of this study was to evaluate the cytotoxic and antitumor activity of spider venom (SV).

Methods: Cell proliferation and cytotoxicity were determined by ³H-methyl thymidine incorporation ([³H]-TDR) assay. DNA fragmentation and cell cycle kinetics were analyzed by FACS. *In vivo* inhibition of tumor size of nude mice by SV (5.0, 10.0, 20.0 mg/kg mice) was constructed.

Results: SV exhibited significant anti-cancer effects on human squamous esophageal carcinoma cells TE13, mainly as a result of cell apoptosis induced by SV. The anti-cancer effects were likely achieved through decreasing [³H]-TdR. TE13 cells treated with SV (25, 50, 100 µg/mL), which were arrested in the G₀/G₁ phase. SV treatment leads to anti-proliferation effects, and significant apoptosis in TE13 cells with reactive oxygen species (ROS) levels can increase dramatically and decrease cellular mitochondrial membrane potential (MMP). In addition, Western blotting analysis showed that one of the pharmacological mechanisms of SV was to activate the expression of P21. *In vivo* testing revealed that tumor size was significantly decreased after 21 days of treatment with the venom ($P < 0.01$).

Conclusions: Our data showed that SVs could inhibit TE13 cell proliferation *in vitro* and *in vivo*.

Introduction

The incidence of human squamous esophageal carcinoma has been increasing in China. Patients with esophageal carcinoma have depended upon surgical resection for cure or prolongation of life. Adjuvant therapy has not significantly altered the outcome of patients with esophageal carcinoma after it has spread beyond the stomach.^{1,2}

Spider venoms (SVs) consist of compounds including ion channel toxins, novel non-neurotoxins, enzymes, and low molecular weight compounds.³ The main toxin components of most SVs have been identified as a complex mixture of proteins, polypeptides, polyamine neurotoxins, nucleic acids, free amino acids, monoamines, and inorganic salts, which are dominated by disulfide-rich peptides that typically have high affinity and specificity for particular subtypes of ion channels and receptors.^{4,5}

In this study, we developed an *in vivo* and *in vitro* evaluation assay to establish that characterization of SVs in human esophageal cancer. This study defines interdependent effects of cells expressing in esophageal cancer, and possible mechanisms have been evaluated.

Materials and methods

Reagents

The human esophageal cell line TE13 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640 medium (Gibco BRL, Life Technologies Inc, Gaithersburg, MD, USA), was dissolved in double distilled water and the pH value adjusted to 7.0 with NaHCO₃, disinfected, and stored at -20°C. Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Co. in

Hangzhou, China), sterilized, and stored at -20°C . LDH was purchased from Sigma Chemical Co, St Louis, USA. Pure spider *Macrothele raveni* venom was collected by electrical stimulation of 15 spiders *M. raveni* (the weight of each spider was approximately 60 g). SV was dissolved in phosphate buffered saline (PBS) and centrifuged at 12 000 rpm for 10 minutes to remove insoluble materials. The concentration of SV was adjusted to 0, 25, 50, and 100 $\mu\text{g}/\text{mL}$. The SV was freeze-dried and stored at -80°C until required.

Cell proliferation assay

The DNA synthesis rates were measured by the method of ^3H -methyl thymidine incorporation (^3H -TdR). Proliferating TE13 cells were seeded onto 96 well plates and incubated for 16 hours. TE13 cells were treated with different concentrations of SV (25, 50, and 100 $\mu\text{g}/\text{mL}$) for the next 24, 48 or 72 hours. ^3H -TdR (1 $\mu\text{Ci}/\text{mL}$) was added and TE13 cells were exposed to ^3H -TdR for 16 hours. The cells were then washed three times with PBS, and lysed with 1 M NaOH. The cells were then harvested onto glass fiber filters with an automatic harvester. Filters were dried and the radioactivity was quantified by liquid scintillation (Beckman counter, USA). Numbers of cells incorporating ^3H -TdR are expressed as percentages of counts of untreated TE13 cells. Results were expressed as percentages of untreated cell counts.

Cell cycle distribution by flow cytometry (FCM)

TE13 cells were treated with 0, 25, 50, and 100 $\mu\text{g}/\text{mL}$ SV, respectively. After incubation for 48 hours, cells were collected and fixed with 70% ethanol at 4°C for 24 hours, then stained with 50 $\mu\text{g}/\text{mL}$ propidium iodide (Sigma). The cell cycle distribution from 10 000 cells was collected using the Epics flow cytometer (Coulter Electronics, Fullerton, USA). Cell cycle analysis was carried out by Multicycle software (Phoenix Flow Systems, San Diego, USA).

Detection of reactive oxygen species (ROS) production

Reactive oxygen species (ROS) production was measured with the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA , Invitrogen), which detects hydrogen peroxide, peroxy radicals, and peroxy nitrite anions. TE13 cells were cultured for 24, 48 or 72 hours with vehicle (control), SV (50 $\mu\text{g}/\text{mL}$) or SV (50 $\mu\text{g}/\text{mL}$) + NAC (10 mM). After 24, 48 or 72 hours, cells were washed with Dulbecco's phosphate-buffered saline (D-PBS). ROS production was measured with the FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA).

Mitochondrial membrane potential

After SV treatment, 40 nM DiOC₆ was added to the cells. Detached cells were pooled together with trypsinized cells and centrifuged at 1000 rpm for eight minutes at 4°C . The cell pellets were washed once with PBS before resuspending in 500 μL PBS containing 40 nM DiOC₆. Fluorescence intensities of DiOC₆ were analyzed by flow cytometry with excitation and emission settings of 484 and 500 nm, respectively. Immediately before flow cytometry analysis, PI (final concentration 30 $\mu\text{g}/\text{mL}$) was added to gate out dead cells.

Analysis of P21 protein by Western blot

Cells were harvested by scraping them into 200 μL of lysis buffer (135 mM NaCl, 25 mmol/L β -glycerophosphate, 20 mmol/L Tris, 2 mmol/L EDTA, 2 mmol/L Na₂P₂O₇, 2 mmol/L DTT, 1 mmol/L Na₃VO₄, 10% glycerol, 1% Triton X-100, 2 $\mu\text{g}/\text{mL}$ aprotinin, and 5 $\mu\text{g}/\text{mL}$ leupeptin [pH 7.5]). The protein content was determined using the Bradford method. Proteins were separated electrophoretically by SDS-PAGE and transferred onto nitrocellulose membranes by semidry blotting. Membranes were incubated with primary and secondary antibodies, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody according to standard procedures, and protein-antibody complexes were visualized with enhanced chemiluminescence (Amersham Buckinghamshire, UK).

Reduction in tumor size in nude mice treated with spider venom (SV)

A xenograft human-nude mouse model was constructed. TE13 cells (5×10^6) were injected subcutaneously at the right dorsal flank of the nude mice. The mice were randomly placed into four groups of six mice each, with the control group receiving only distilled water (0 μg SV/g mice) each day as a placebo. The three treated groups were inoculated with TE13 tumor cells and received SV (5.0, 10.0, 20.0 mg/kg mice) by tail vein injection. Every three days we measured the size of tumors, and at all points of analysis thereafter, for 21 days. Tumor volume was evaluated by caliper measurements and was calculated as length \times width \times depth.

Statistical analyses

Results were expressed as mean \pm SD. ANOVA and the protected Fisher's Least Significant Difference were used for analysis. Differences between values were considered significant at $P < 0.05$.

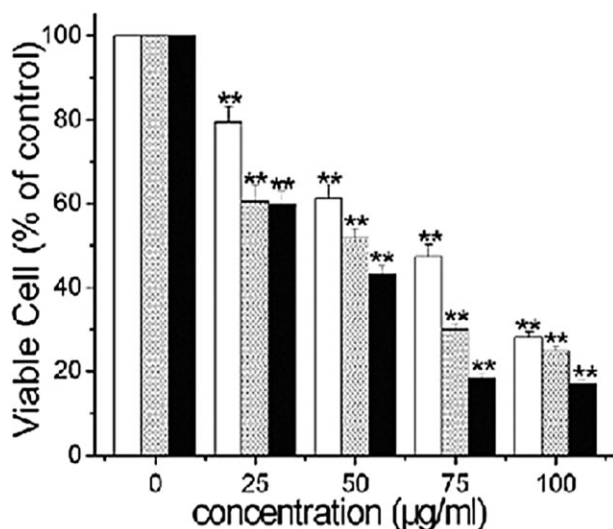


Figure 1 Synthesis of DNA in TE13 cells was inhibited by spider venom (SV) using [^3H]-TdR incorporation assay. □, 24 h; ▨, 48 h; ■, 72 h.

Results

The effect of SV on [^3H]-TdR incorporation in TE13 cells

Synthesis of DNA in these cells was inhibited. SV caused marked dose- and time-dependent DNA inhibition in TE13 cells, at concentrations ranging from 25 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$ by liquid scintillation (Beckman counter, U.S.A) ($F[4,21] = 86.99$, $P < 0.01$). SV significantly decreased cell proliferation. The SV-induced inhibition of proliferation was also time-dependent inhibition in TE13, at times ranging from 24, 48, and 72 hours ($F[3,11] = 89.88$, $P < 0.01$) (Fig. 1).

The cells were then treated with [^3H]-TdR for 12 hours, with IC_{50} ranging from 50 to 70 $\mu\text{g/mL}$ after 24, 48, and 72 hours. The degree of growth inhibition of TE13 in the presence of SV was dose dependent. SV significantly decreased cell proliferation, but the decrease differed with the different concentrations. Results are expressed as % of control ($n = 6$. Mean \pm SD. $^{**}P < 0.05$, $^{***}P < 0.01$ vs. control group).

Inhibition of cell cycle progression and induction of apoptosis

As shown in Table 1, SV caused a significant dose-dependent accumulation of TE13 cells in the G_0/G_1 phases, decreased in the G_2/M and S phases from 25 to 75 $\mu\text{g/mL}$ at 48 hours. The differences in cell cycle distribution between vehicle-treated TE13 cells and 25, 50, and 100 $\mu\text{g/mL}$ treated are statistically significant ($P < 0.01$) (Table 1). Increased cell cytotoxicity occurred with increased concentrations of SV. The effect of

Table 1 Effect of spider venom on cell cycle distribution in TE13 cells. $n = 6$. Mean \pm SD. $^{\text{B}}P < 0.05$, $^{\text{C}}P < 0.01$ versus control

Group ($\mu\text{g/mL}$)	Cell cycle distribution (%)		
	G_0/G_1	S	G_2/M
Control	40.5 ± 2.1	32.9 ± 1.9	26.9 ± 1.1
Treated			
25	$50.6 \pm 2.0^{\text{B}}$	$20.3 \pm 3.9^{\text{C}}$	$29.8 \pm 2.4^{\text{C}}$
50	$61.2 \pm 3.2^{\text{B}}$	$18.8 \pm 4.4^{\text{C}}$	$20.5 \pm 2.2^{\text{C}}$
100	$69.7 \pm 4.0^{\text{B}}$	$14.5 \pm 2.7^{\text{C}}$	$15.2 \pm 1.9^{\text{C}}$

SV on TE13 cells was analysed by fluorescein-labelled annexin V- and PI-stained cells. The ratio of apoptosis and necrosis of the cells was increased (Fig. 2, Table 2).

SV induced apoptosis is associated with ROS increase

In the current study, we examined the effect of SV exposure on the production of ROS. The results showed that exposure of SV (50 $\mu\text{g/mL}$) and TE13 cells led to a significant increase in the levels of ROS production for 24, 48, and 72 hours (Fig. 3). The involvement of the SV-induced increase in ROS generation in apoptosis was further confirmed using the ROS scavengers NAC. The results demonstrated that treatment with ROS scavengers led to a significant reduction in SV-induced TE13 cell killing and apoptosis. Furthermore, the degree to which the ROS scavengers prevented the effects of SVs on tumor cell killing directly correlated with the ability of the scavengers to reduce the level of SV-induced ROS.

The effect of ROS scavengers on SV-induced cell toxicity was determined. TE13 cells were cultured for 24, 48, and 72 hours with vehicle (control), SV (50 $\mu\text{g/mL}$), or SV (50 $\mu\text{g/mL}$) + NAC (10 mM). FACS determined the viable cell number. The data represent the mean \pm SEM of triplicate cultures.

SV induced apoptosis is associated with changes in mitochondria

The mitochondrial membrane potential (MMP) level was detected to determine possible involvement of mitochondria

Table 2 Effect of spider venom on cell apoptosis rate in TE13 cells. $n = 6$. Results are expressed as % of control. $^{\text{B}}P < 0.05$, $^{\text{C}}P < 0.01$ versus control

Group ($\mu\text{g/mL}$)	Apoptosis rate (%)	Necrosis rate (%)
Control	0.45 ± 0.07	0.2 ± 0.0
Treated		
25	$8.02 \pm 1.9^{\text{B}}$	$1.3 \pm 0.7^{\text{B}}$
50	$14.1 \pm 2.2^{\text{C}}$	$6.2 \pm 1.1^{\text{B}}$
100	$21.8 \pm 2.3^{\text{C}}$	$7.1 \pm 2.0^{\text{B}}$

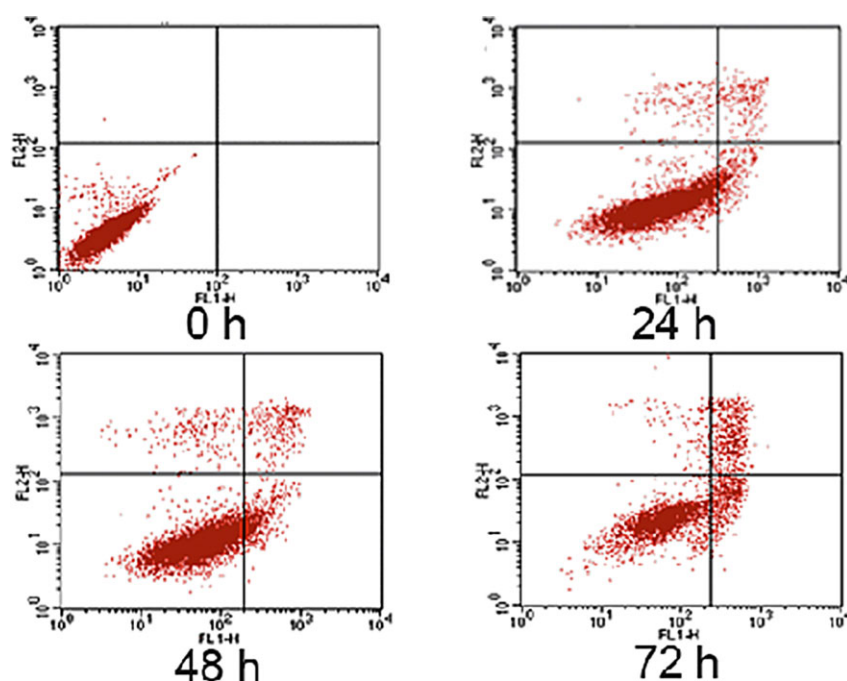


Figure 2 Flow cytometry histograms of apoptosis assays by the propidium iodide (PI) method. TE13 cells were treated with 50 µg/mL of spider venom (SV) overnight.

in SV-induced cell death. Exposure of TE13 cells to SV resulted in a decreased MMP level (Fig. 4). As shown in Figure 4, the changes in MMP induced by SV were completely inhibited by pre-treatment to TE13 cells.

Effects of SV on the activity of P21 protein

TE13 cells treated with SV for 24 hours were analyzed for the P21 protein activity by Western blot. The expression of P21 showed enhanced activity after SV treatment in TE13 cells, which became four times higher than those in the control cells (Fig. 5).

In vivo reduction in tumor size in nude mice using SV

In mice treated with SV, concentrations ranged from 0,5,0,10,0,20,0 mg/kg mice. Twenty-one days after tail vein

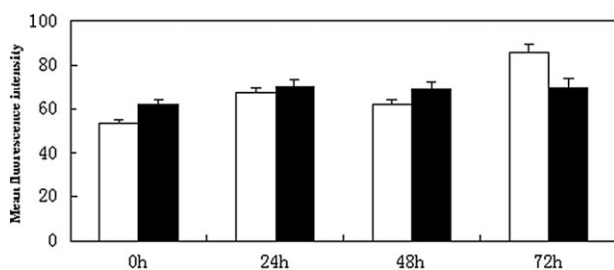


Figure 3 The effect of spider venom (SV) on the production of reactive oxygen species in TE13 cells. □, SV; ■, SV + NAC.

injection, growth of TE13 tumors was significantly reduced as compared with that seen in the control mice ($P < 0.05$, Table 3).

Discussion

To harm or kill tumors requires agents that will kill cells. Two types of cell death are necrosis and apoptosis. Apoptosis is a

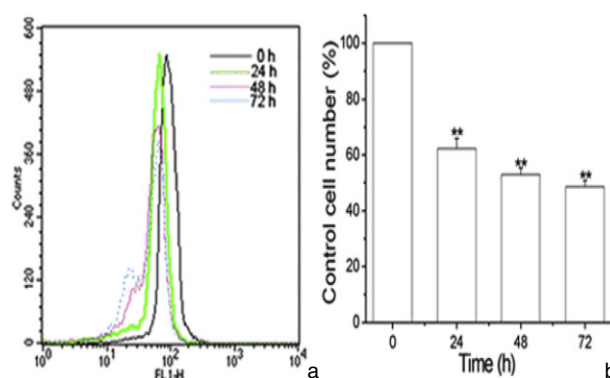


Figure 4 The effect of spider venom (SV) on mitochondrial membrane potential (MMP) of TE13 cells. (a) Fluorescence intensity was analyzed by flow cytometry with excitation and emission settings of 484 and 500 nm, respectively. Panels a–f were obtained with TE13 cells treated for 24, 48, and 72 hours with 50 µg/mL of SV and the changes in MMP were measured. (b) The percentage shown is the reduction in the treated cells compared with the control.

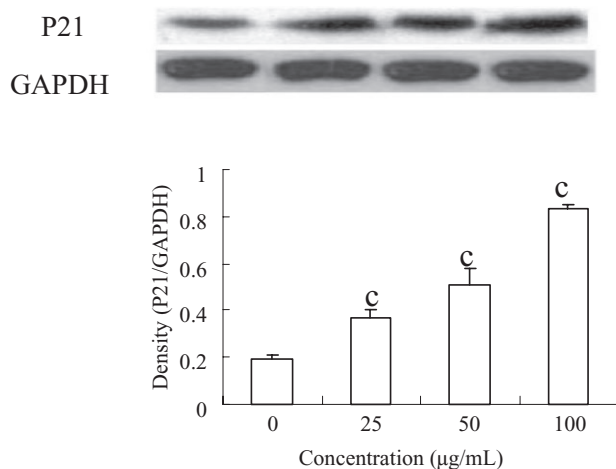


Figure 5 The expression of P21 was up-regulated in response to spider venom by Western blot analysis TE13 cells (a). The densitometric analysis of the levels of P21 by an image analysis system is shown (b). ($n = 6$. Mean \pm standard deviation [SD]. ^C $P < 0.01$ vs. control group). ANOVA with subsequent multiple comparisons test.

programmed cell death, which is a regulatory process of cell self-destruction critical for metazoan survival. Necrosis is cell death by injury. A critical stage of apoptosis involves the acquisition of surface changes by dying cells that eventually result in the recognition and the uptake of these cells by phagocytes.^{6,7} In general, necrotic cells swell and lyse, whereas apoptotic cells shrink and fragment. Cytotoxic effects against a tumor cell panel have been well documented elsewhere.^{8,9} The apoptotic portion of cell death was cell cycle-dependent and associated with changes in carcinoma cells.^{10–12}

The above results indicate that at high concentrations, SV induced cell death in cultured TE13 cells. Apoptosis was observed in approximately 20% of the lines, but the main form of cell death, however, was necrosis (Fig. 2, Table 2). *In vitro* experiments on morphology and biochemistry revealed typical apoptotic characteristics and led features characteristic of apoptosis following treatment. [³H]-TdR assay showed that cell viability was inhibited by concentrations in the ranges of 0, 25, 50, and 100 µg/mL. Treatment with venom

significantly increased cytotoxicity determined by [³H]-TdR release in TE13 cells. Results showed that SV exerted necrosis and apoptosis (Table 3) to kill TE13 cells. Two forms of cell death were that during the initial stages of apoptosis the cell membrane remained intact, while at the moment that necrosis occurred, the cell membrane lost its integrity and commenced leaking.

Here, through flow cytometry (FCM) assay, we found, in comparison with cell control, cell apoptosis increased markedly in treated cells, while the cell cycle significantly accumulated in the G₀/G₁ phase. It is a very interesting result. In order to elucidate the pathway leading to the change of cell cycle and apoptosis, we examined the activation of P21, which was reported to initiate apoptosis upon various stimuli. P21 is a cyclin-dependent kinase. Some studies have demonstrated that the target gene in the cells and their differentiation was well related to the transcriptional activation of the p21 gene.^{13,14} We examined activation of P21 by Western blot. The results showed that the expressions of apoptosis associated protein P21 were up-regulated. This suggested that the anti-tumor effect of SV on TE13 cells was related to the induction of apoptosis after changing the cell cycle. Subsequently, the results suggested that the anti-tumor effect of SV on TE13 cells was related to the induction of apoptosis and necrosis, which have demonstrated that mitochondrial alterations constitute critical events of the apoptotic cascade. Reduction of MMP is among the changes encountered during the early reversible stages of apoptosis. The decrease in MMP in apoptotic cells is associated with a reduction of ROS uptake.

Conclusion

In conclusion, it is likely that for the anti-tumor activity of SV to be effective, close contact between SV and tumor cells is important for the development of either apoptosis or necrosis of TE13 cells. Apoptosis, necrosis, and lysis of tumor cells are possible mechanisms by which the drug inhibited tumor growth. *In vitro* and *in vivo* assays on human cervical carcinoma cell line (HeLa),¹⁵ hepatocellular carcinoma cell line (BEL-7402),¹⁶ and human lung adenocarcinoma¹⁷ suggested that SV facilitates apoptosis and functions as an effective anti-cancer agent. Further, more structural aspects responsible for these activities need to be defined, and the results of more advanced *in vivo* tests will be pivotal in contemplating SVs further development as a clinically useful pharmaceutical agent.

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Table 3 Effect of spider venom on tumor volume (length \times width \times depth) in nude mice

Group (mg/kg mice)	Number of nude mice	Volume of the tumor (mm ³)
Control	6	39.7 \pm 1.63
Treated		
5.0	6	13.9 \pm 0.54 ^B
10.0	6	6.6 \pm 0.41 ^B
20.0	6	2.7 \pm 0.36 ^B

$n = 6$. Mean \pm standard deviation (SD). ^BValue was different $P < 0.05$ from corresponding value in untreated mice.

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Disclosure

No authors report any conflict of interest.

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