The agkistrodon acutus venom componets of X in vitro anti-tumor effect and mechanism

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Abstract

Objective To observe the agkistrodon acutus venom componets of X(FX) on human colonic adenocarcinoma cell HCT-8 inhibition and to explore its mechanism. **Method**: From the cells, protein and gene of the three levels of the agkistrodon acutus venom componets of X(FX) of HCT-8 cell inhibition and to explore its anti-tumor. mechanism. Using colony formation assay detection of the agkistrodon acutus venom components of X of HCT-8 cell proliferation effect, using RT-PCR detection of apoptosis-related genes Caspase, survivin, Bax, bcl-2mRNA level changes, using flow cytometry FX After the effects of different time, HCT-8 cells, Caspase-3 protein and Survivin protein expression. **Results**: FX on the HCT-8 cells significantly inhibited the proliferation, FX up-regulated caspase-3, reduced survivin, Bax, bcl-2mRNA gene expression, FX acting on the HCT-8 cells, Caspase-3 protein expression increased and then gradually decreased to below normal, Survivin protein in the role of PF-II expression after 6-48 h sustained reductions in. **Conclusion** The agkistrodon acutus venom components of X can be raised to promote apoptosis-related genes, down-induced apoptosis genes inhibited apoptosis in HCT-8. [Life Science Journal. 2010; 7(1): 41 – 45] (ISSN: 1097 – 8135).

Key words: apoptosis; gene; the agkistrodon acutus venom; anti-tumor

Anti-tumor effect of snake venom has gradually been recognized and applied, however anti-tumor mechanism of snake venom is not yet clear. We extracted from the agkistrodon acutus venom anti-tumor componets and to research its anti-tumor mechanism.

1. Materials and methods:

Main materials: The agkistrodon acutus venom componets of X(FX),in our laboratory using monoclonal antibody technology extraction, Purity of more than 95%; HCT-8 cells, this experiment preservation; Caspase3 McAb polyclonal antibodies, rabbit anti-human survivin monoclonal antibody (Santa Cruz, USA), RNA inhibitor (Promega, USA), RNA enzymes (Sigma, USA).

Methods:

1.2.1 Colony formation assay

Logarithmic growth phase of the HCT-8 tumor cells, With 0.25% trypsin digestion,.and made into single cell suspension count, transfer cell concentration of 3 × 103/ml, were inoculated on 24-well plate (3 × 102 / hole), cells cultured 16 h to be fully adherent to join after the snake venom and its components X, to continue to develop 72 h, supernatant was washed two times with culture medium, to continue to foster exchange of fresh culture medium 7 ~ 14 d. Methanol at room temperature a fixed 15 min, Giemsa stain at room temperature 30 min, tap water wash away the residual dye liquor, counting colonies (including more than 50 cells, namely, diameter ≥ 0.5 mm of the cell colony) number, and calculate the average colony number of each group.

1.2.2 Determination of apoptosis-related genes

(1) primer design

According to four kinds of gene mRNA sequences, are designed for caspase-3, bcl-2, GAPDH, Bax, and survivin, PCR primers (designed by Shanghai Bio-engineering and synthetic.)

caspase-3 primers
Forward 5' TTT TTC AGA GGG GAT CGT TG 3 '
Backward 5’ GCC TCC ACT GGT ATT TTA TG 3’
bel-2 primers
Forward5’CCA GAT CCC AGA GTT TGA GC 3’
Backward 5’ATG ATG GCT GCT GCT GGT TG 3’ 29
GAPDH primers
Forward5’TAT TGG GCG CCT GGT CAC CA 3’
Backward 5’ CCA CCT TCT TGA TGT CAT CA 3’
Bax primers
Forward5’ TTT GCT TCA GGG TTT CAT CC 3’
Backward 5’ CAG TTG AAG TTG CCG TCA GA 3’
survivin primers
Forward5’ GGA CCA CCG CAT CTC TAC AT 3’
Backward 5’ GAC AGA AAG GAA AGC GCA AC 3’

(2) Tumor cell total RNA extraction and quantification

(a) Equipment ready: Plastic equipment with 1% DEPC distilled water soak1-2 h after the high-pressure sterilization, back to dry, Glass equipment at 180 ℃ high temperature dry roasted 5 hours standby

(b) Cell preparation: Logarithmic growth phase HCT-8 cells, with 0.25% trypsin digestion, made into single cell suspension counted, transfer cell concentration of 1 × 106/ml, inoculated 6-well plate (5 × 105 / hole), cultured 16 h to be fully adherent cells , 400μg/ml of each hole to join the PF-II, final volume of 1.5 ml, 0,2,6,12,24 and 48 h post-training,Cells were collected, respectively, using Trizol kit from the total RNA, reverse transcriptase synthesized DNA, synthesized by the PCR double-stranded DNA, while synthesis of mouse glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as internal reference.

(c) Trizol extraction of total cellular RNA: the collection of cells, transferred to 1.5 ml eppendorf tube, add 800μl Trizol, a gun repeatedly pumping blending, at room temperature 5 min, by adding 200μl chloroform volatility in 30 sec, room temperature for 3 min,12,000 rpm centrifugation at room temperature 5 min, the supernatant absorption to another 1.5 ml eppendorf tube, add an equal volume of isopropyl alcohol mixing at room temperature 30min, centrifuged 12,000 rpm at room temperature 5 min,supernatant was washed with 70% ethanol twice, centrifuged 12,000 rpm at room temperature 2 min, discard supernatant, vacuum drying, precipitation dissolved in 50μl DEPC water.

(d) RNA Quantitative: Lessons 2μl RNA, 50-fold diluted by UV spectrophotometer OD260 and OD260/OD280 value, OD260/OD280 value between 1.8 to 2.0, indicating RNA of high purity, this time, 1 OD is equivalent to 50μg/ml double-stranded DNA , 40μg/ml single-stranded DNA or RNA. RNA concentration determined by the formula: Concentration (μg / ml) = OD260 × dilution factor × 40, quantitative after -70 ℃ to save.

(3) RT-PCR method

2μg RNA,1μl RNase inhibitor(20 units/μl),3μl 25 pmol/L Oligo dT,2μl 10 mmol/L dNTP,4μl 25 mmol/L Mg2+,0.5μl AMV(10 units/μl), Oligo dT, 2μl 10 mmol / L dNTP, 4μl 25 mmol / L Mg2 +, 0.5μl AMV (10 units / μl), 42 ℃, 60 min after 95 ℃, 5 min inactivation of reverse transcriptase enzymes and destruction of mRNA-DNA hybrids, ice bath 5 min after PCR amplification. PCR reaction system containing both ends of each primer, 25 pmol, reverse transcriptase product of 5μl, Taq enzyme 1μl (5unit/μl). Reaction conditions: 94 ℃ 1 min, 57 ℃ 1 min, 72 ℃ 90 sec, 35 cycles after the end of the last cycle, 72 ℃ extension of 10 min, 1% agarose gel electrophoresis, PCR analysis of the results of UV lamp.

(4)Agarose gel electrophoresis and gray-scale analysis

With 0.5 × TBE running buffer (0.045 mol/LTris-boric acid, 0.001 mol / LEDTA) with 0.8 ~ 1.2% (W / V) agarose gel electrophoresis, liquid, heated to agarose completely dissolved, add EB (10 mg / ml) to a final concentration of 0.5μg/ml; sealing a good adhesive, after mixing into the mold, insert the appropriate comb, comb of about 1.0 mm from the floor until the glue completely solidified, crefully remove the comb, the gel into the electrophoresis containing 0.5 × TBE electrophoresis buffer tank,take apropriate sample and a proper volume of loading buffer (0.25% bromophenol blue, 0.25% xylene blue FF, 40% sucrose) blending, mcro-plus with the comb-like device added to the hole to 5 V / cm voltage electrophoresis, when the electrophoresis to the appropriate location, in the long-wave UV lamp observations and photographed.After the photographed analyzed with the purpose of image analysis of the gray zone and GAPDH, and calculate the ratio between the two.

1.2.3 FCM detection of Survivin and Caspase-3 protein expression

(1) cell culture

Logarithmic phase were collected from HCT-8 cells were treated with 0.25% trypsin digestion，made into single cell suspension, counting, transfer cell concentration of 1 × 106/ml, were inoculated on 6-well plate (5 × 105 / hole),cells cultured 16 h to be fully adherent, each hole by adding 400μg/ml of the PF-II, final volume of 1.5 ml, cultured 0,6,12,24 and 48 h respectively after the cells were collected, fixed in 70% cold ethanol, and placing -20 ℃ preservation within a week with the FCM assay.

(2) monoclonal antibody

Washing the cells with PBS twice, plus an anti-(rabbit anti-Caspase-3, Santa Cruz Company, USA) 3150μl (1:50 dilution) at 4 ℃ for the role of 45 min,plus serum (1:20 dilution) 50μl closed 30 min, PBS washed cells twice, plus wo anti-(FITC tag goat anti-rabbit IgG, Santa Cruz Company, USA) 50μl (1:100 dilution) at 4 ℃ for the role of 45 min, PBS washed cells twice.

(3) protein expression detected by flow cytometry

Add 300μl PBS, protein expression detected by flow cytometry, 104 cells per sample collected, Cellquest software analysis. Results The positive cells expressed as a percentage.

1.2.4 Statistical Methods

Two samples were used to compare the number of Student's t test.

2 Results

2.1The agkistrodon acutus venom componets of X on cell HCT-8 colony formation.

Test results showed that when the concentration of 400μg/ml, 800μg/ml, when, FX process after 72 h, HCT-8 cells, the number of colony formation were significantly reduced compared with control group were significantly different (respectively P <0.01). Showed that snake venom and its components on the HCT-8 colon adenocarcinoma cells significantly inhibited the proliferation. The results shown in Table 1 and Figure 1.

2.2 The snake venom component X on the HCT-8 cells, apoptosis-related genes.

Using RT-PCR detection of mRNA level changes. The results showed that, FX process after 2 ~ 12h, HCT-8 cells, caspase-3 mRNA on average higher than that of water. The results in Table 2.

Table 1. Effect of snake venom and traction X on colony formation of HCT-8 cells

Group	Concentration ()	Number of colonies	Rate of colony formation (%)
Control	0	199±7.35	100
SV	400	173±6.98	86.9
Fraction X	400	132±9.83**	66.3
Control	0	197±6.91	100
SV	800	196±7.24	99.5
Fraction X	800	69±6.47**	35.3

**P<0.01, vs control

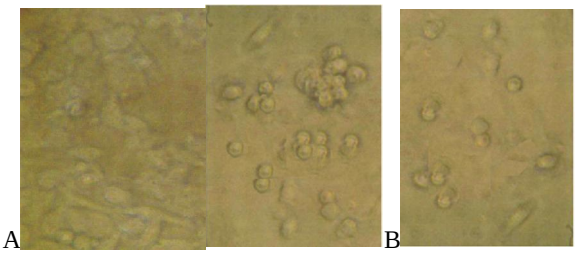

Figure 1. Venom X-component of the HCT-8 cell line A as the control group, B is 400ug/ml, C for 800ug/ml (× 40)

Table 2. Changes in Caspase-3 mRNA level of HCT-8 cells at different time points after treatment with 400μg/ml FX

Time(h)	Ratio%
0	0.93±0.02
2	1.27±0.01
6	1.11±0.04
12	1.08±0.02

2.2.2 snake venom components FX pairs of HCT-8 cells, Bax mRNA levels in

Using RT-PCR detection of mRNA level changes. The results showed that, PF-II process after 2 ~ 24h, HCT-8 Cells, Bax mRNA levels decreasing. The results in Table 3.

2.2.3 snake venom components FX pairs of HCT-8 cells, bcl-2 mRNA expression in

Using RT-PCR detection of mRNA level changes. The results showed that, FX process after 2h, HCT-8 cells, bcl-2 mRNA levels began to decrease, the role of post-24h, bcl-2 mRNA levels reduced to minimum. The results in Table 4.

2.2.4 snake venom components FX pairs of HCT-8 cells, expression of survivin mRNA

Using RT-PCR detection of mRNA level changes. The results showed that, FX process after 2 ~ 24h, HCT-8 cells, survivin mRNA were lower than the control group. The results in Table 5.

Table 3. Changes in Bax mRNA level of HCT-8 cells at different time points after treatment with 400μg/ml FX

Time(h)	Ratio%
0	0.94±0.04
2	0.73±0.04
6	0.92±0.04
12	0.58±0.02
24	0.78±0.04

Table 4. Changes in bcl-2 mRNA level of HCT-8 cells at different time points after treatment with 400μg/ml FX

Time(h)	Ratio%
0	1.635±0.055
2	1.290±0.030
6	1.120±0.060
12	1.005±0.025
24	0.840±0.050

Table 5. Changes in survivin mRNA level of HCT-8 cells at different time points after treatment with 400μg/ml FX

Time(h)	Ratio%
0	1.31±0.03
2	1.09±0.04
6	1.07±0.03
12	0.96±0.02
24	1.17±0.04

Table 6. Changes in Caspase-3 protein expression of HCT-8 cells after treatment with 400μg/ml FX

Time (h)	Percentage of positive cells (%)
0	37.29±7.00
6	42.45±6.54
12	20.76±2.43*
24	15.58±1.24**
48	6.09±0.6*

2.3.Apoptosis-related changes in protein expression

2.3.1 snake venom components FX pairs of HCT-8 cells, Caspase-3 protein expression

FX effect detected by flow cytometry at different times after, HCT-8 cells, Caspase-3 protein expression changes. The results show that, FX process after 6 h, HCT-8 cells, Caspase-3 protein expression increased and then gradually decreased to below normal levels. The results in table 6.

2.3.2 snake venom components FX pairs of HCT-8 cells, Survivin protein expression

FX effect detected by flow cytometry at different times after, HCT-8 cells of Survivin protein. The results show that, FX process after 6 h, HCT-8 cells, Survivin protein expression began to decrease continued lower ($P < 0.05$), 48 h to a minimum value ($P < 0.01$). The results in Table 7.

Table 7. Changes in Survivin protein expression of HCT-8 cells at differ points after treatment with 400μg/ml FX

Time(h)	Percentage of positive cells (%)
0	42.20±3.99
6	31.74±5.75
12	34.53±2.85
24	27.17±6.99*
48	12.29±1.43**

*P<0.05,**P<0.01 vs 0 h

3. Discussion

Venom from the snake's venom gland secretion of a natural toxic protein complex composition, mainly of protein, peptide, and a number of enzymes, with a wide range of biological activity, can be used as a natural medicinal resources. As the pharmacology, toxicology, pharmacology development of many components of snake venom have been a more in-depth study and found that their anti-thrombosis, hemostasis, analgesic and anti-tumor aspects of great potential.

The exact anti-tumor mechanism of snake venom has yet to clear, the current point of view include: interference with membrane transport mechanisms; affect cellular energy metabolism; affect the body's immune system; Inducing Interferon; affect the blood hypercoagulability; -induced apoptosis; inhibit angiogenesis [1-7], In this study, cells, proteins genes on the, the agkistrodon acutus venom componets of X of three levels induced apoptosis in the mechanism study.

Apoptosis is an extremely complex and sophisticated process, involving many events, including the expression of apoptosis-related genes such as increased or reduced. In general apoptosis need to undergo initiation, effector and degradation period of three stages. In the initial stage, the cells are from outside of the various types of apoptotic signals stimulated a series of biochemical reactions; In effect stage, the cells showed a variety of biochemical reactions to form one or more of the signal transduction pathway; Finally, the cells enter the degradation phase, performance for a variety of hydrolytic enzymes (including the specific protease Caspases and nucleic acid enzymes) are activated, cell DNA degradation, nuclear chromatin condensation, cells membrane permeability and cell surface molecules change, as well as the cytoplasm of reactive oxygen substances (reactive oxygen species, ROS) increased and a series of apoptosis. Has confirmed that apoptosis occurs mainly through two ways. First, receptor-mediated pathway, from receptor-mediated activation of Caspase-8 launched the protease cascade to apoptosis; The other one is the mitochondrial pathway by the mitochondrial release of cytochrome C-mediated activation of Caspase-3 induced apoptosis [8]. Endogenous pathway, cytochrome C release in the center of apoptosis. Stage of apoptosis, due to internal and external mitochondrial membrane permeability change, led to disappearance of mitochondrial membrane potential and cytochrome C and other pro-apoptotic factor release, these pro-apoptotic factor released into the cytoplasm can lead to apoptosis cascade reaction, including Caspase-3 and other substances in activation of proteolytic enzymes and DNA fragments appear, in this process, the mitochondrial membrane permeability transition pore (PTP) opening and closing is to determine whether the cells the key to apoptosis [9]. But Bcl-2 family members on the regulation of apoptosis mainly through regulation of mitochondrial PTP opening and closing to achieve. Bcl-2, Bcl-xL inhibited the PTP opening such as to inhibit the release of apoptotic factors and prevent the occurrence of apoptosis, but Bax, Bad, Bak, Bik, Bcl-xS etc. as the role of the contrary, hey promote PTP opening, thereby promoting the release of apoptotic factors.

Cells in Bax protein can form a homodimer their own,and easily Bcl-2 protein in the formation of heterodimer complex, So that Bax inactivation. Bcl-2 expression increased when the relative increase in Bcl-2/Bax dimers, make the elimination of the pro-apoptotic effect of Bax, but the relative increase in expression of Bax, when Bax / Bax homodimers increase, Increase pro-apoptotic effect. This study is based on snake venom-induced apoptosis of tumor cells results, selected two kinds of human tumor cells in colon adenocarcinoma cell line HCT-8 and ovarian cancer cells SKOV-3 pathway in the regulation of the PTP opening and closing of two key factors Bax and bcl-2 gene, downstream ffector caspase-3 and a newly discovered inhibitor of apoptosis gene survivin levels of mRNA detection, as well as one of the pro-apoptotic gene caspase-3 and apoptosis inhibiting gene survivin protein levels were detected,to discuss the possible mechanism of snake venom-induced apoptosis of tumor cells. China have not seen the similar report on the the agkistrodon acutus venom-induced molecular mechanisms of tumor cell apoptosis. We have detected that the HCT-8 cells treated with FX at different times after treatment, apoptosis promoting gene Bax, caspase-3 and apoptosis-suppressing gene Bcl-2, survivin transcription level changes. The results showed that, FX dealing with HCT-8 cells after 2-24 h, Bax mRNA level was no higher, Caspase-3 mRNA levels in the PF-II dealing with 2-12h showing a rising trend, pro-apoptotic gene expression increased; At the same time, suppressing apoptosis gene bcl-2 mRNA levels in the 2-24h after treatment showed sustained reductions in trend, survivin mRNA levels at all time points were lower than the control group, and at 2-12h after treatment continued to decrease, that is, reduced gene expression of apoptosis suppression. These results suggest that, FX can induce increased expression of certain pro-apoptotic genes, the suppression of apoptosis gene expression decreased, and thus can promote the HCT-8 tumor cells apoptosis, thus inhibit tumor cell growth. Furthermore we were selected one of the pro-apoptotic gene and an inhibition of apoptosis gene, that is pro-apoptotic gene caspase-3 and apoptosis inhibiting gene survivin, using fluorescent antibody staining by FCM to detect Caspase-3 and Survivin protein expression levels.The results showed that, FX acting on the HCT-8 cells after 6 h, Caspase-3 protein expression increased, and then gradually decreased to normal levels below, Survivin protein in the role of PF-II expression after 6-48 h sustained reductions ($P < 0.05 \sim P < 0.01$),note the agkistrodon acutus venom inhibit the growth of HCT-8 tumor cells through the regulation of apoptosis-related gene expression achieved. Based on the above results suggested that, the agkistrodon acutus venom componets of the role of HCT-8 cells, by down-regulating bcl-2 expression to make irreversible PTP opening, outer mitochondrial membrane of small molecules into the membrane, mitochondria transmembrane potential loss, membrane damage, between the inside and outside the membrane leading to mitochondrial cytochrome C and other pro-apoptotic

factor release, the cytochrome C and apoptosis protein-activating factor 1 (Apaf1) combined, activatd Caspase-9, activation of Caspase-3 and then the cells underwent apoptosis, Survivin protein expression in the same time, down to the role of Caspase-3 inhibition reduced, Caspase-3 activity was enhanced cell apoptosis.

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