Acta Physiologica Sinica, February 25, 2007, 59 (1): 58-62
http://www.actaps.com.cn

Research Paper

Effect of the venom of the spider *Macrothele raveni* on the expression of *p21* gene in HepG2 cells

GAO Li[1,*], SHEN Jin-Bao[2], SUN Jie[3], SHAN Bao-En[4]

[1]*College of Life Science, Hebei Normal University, Shijiazhuang 050016, China;* [2]*Huihua College, Hebei Normal University, Shijiazhuang 050091, China;* [3]*Science and Technology Company, Hebei Medical University, Shijiazhuang 050017, China;* [4]*Research Centre, The Fourth Hospital of Hebei Medical University, Shijiazhuang 050011, China*

Abstract: This paper focuses on the effect of the venom of the spider *Macrothele raveni* on the proliferation of human hepatocelluar carcinoma cell line HepG2 and the related molecular mechanism. XTT test showed that the proliferation of HepG2 cells *in vitro* was inhibited by the spider venom ($P<0.05$) in a concentration-dependent manner. By using flow cytometry, it was found that the spider venom caused selective G_2/M cell cycle arrest in HepG2 cells. RT-PCR and Western blot indicated the expressions of *p21* mRNA and protein in HepG2 cells were obviously up-regulated by the spider venom. The venom of the spider *Macrothele raveni* inhibited the proliferation of HepG2 cells. These results suggest that the possible mechanism of the spider venom is to activate the expressions of *p21* gene and protein and to cause selective cell cycle arrest at G_2/M phase, leading to HepG2 cell apoptosis.

Key words: the venom of the spider *Macrothele raveni*; HepG2 cells; cell cycle; *p21* gene; p21 protein

雷氏大疣蛛毒液对人肝癌 HepG2 细胞 *p21* 基因表达的影响

高 莉[1,*]，沈金宝[2]，孙 捷[3]，单保恩[4]

[1] 河北师范大学生命科学学院，石家庄 050016；[2] 河北师范大学汇华学院，石家庄 050091；[3] 河北医科大学科技总公司，石家庄 050017；[4] 河北医科大学第四医院科研中心，石家庄 050011

摘　要：本文研究了雷氏大疣蛛毒液对人肝癌细胞株 HepG2 增殖抑制作用及其分子机制。采用 XTT 法观察到雷氏大疣蛛毒液剂量依赖抑制 HepG2 细胞增殖；流式细胞仪检测发现，经过雷氏大疣蛛毒液作用的 HepG2 细胞周期发生明显的选择性改变；RT-PCR 方法检测到 *p21* 基因表达增强；Western blot 检测发现，p21 蛋白表达增加。结果提示，雷氏大疣蛛毒液抑制人肝癌细胞 HepG2 增殖的可能机制之一是使 *p21* 基因和蛋白表达增加，G_2/M 细胞周期被阻滞，从而诱导细胞凋亡。

关键词：雷氏大疣蛛毒液；HepG2 细胞；细胞周期；*p21* 基因；p21 蛋白
中图分类号：R33

Hepatocellular carcinoma (HCC) is one of four most prevalent malignant diseases in China[1]. The spider *Macrothele raveni* was identified as a new species of genus *Macrothele*[2]. The purification and preliminary test of raventoxin-II in mice showed that it acted as a neurotoxic peptide[3]. Raventoxin-I and -III were isolated and identified as toxins. They can kill mice and block neuromuscular transmission in the isolated mouse phrenic nerve diaphragm preparation, but have no effect on cockroaxches[4]. Venom specificity remained unchanged by chemical modification. The strategy was based on the findings of others concerning the anti-tumor ability of the spider venom. In our study the effect of the spider venom on human HCC HepG2 cell growth and the possible related mechanisms were evaluated.

Received 2006-07-05　Accepted 2006-11-14

This work was supported by the National Natural Science Foundation of China (No. 30371753), the Research Project of Hebei Education Department (No. 2004110) and the Doctor Foundation of Hebei Normal University (No. L2005B25).

[*]Corresponding author. Tel: +86-311-86268655; Fax: +86-311-86268313; E-mail: ligao0823@163.com; gaoli0823@yahoo.com.cn

1 MATERIALS AND METHODS

1.1 *Reagents*

Pure spider *Macrothele raveni* venom was collected by electrical stimulus to 15 spiders (about 60 g). The spider venom was freeze-dried and stored at -80 °C until used. Dissolved in phosphate-buffered saline (PBS) and centrifuged at 1 000 *g* for 10 min to remove insoluble materials, the spider venom was adjusted to the final concentrations of 0, 10, 20 and 40 μg/mL. The test solutions were freshly prepared for different experiments and obtained by diluting the stock solution in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL Gaithersburg, MD, USA). PBS was used as a negative control. Cisplatinum (DDP) was obtained from Sigma Chemical Co. (St Louis, MO, USA).

1.2 *Cell culture*

Human hepatoma (HepG2, ATCC HB8065) cells were cultured with DMEM containing 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L *L*-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in plastic disposable tissue culture flasks at 37 °C in a 5% CO_2/95% air incubator. The cells were first incubated for 2 d in 0.1% FCS and then harvested by centrifugation and washed 6 times and passaged every 2 d.

1.3 *Cytotoxicity assay*

The inhibition of cell proliferation was assessed by sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) (Sigma Chemical Co., St Louis, MO, USA) assay as described by Roehm *et al*.[5] with modification. Briefly, HepG2 cells were subcultured on a 96-well plate with 1×10^4 cells/well in 100 mL medium. After 24 h of incubation, the medium in 96-well plate was discarded and replaced with medium containing different concentrations of the spider venom (10, 20 and 40 μg/mL), DDP (as positive control) or PBS (as negative control). The treated cells were incubated at 37 °C for 48 h, and each well then received 50 mL of XTT test solution prepared by mixing 5 mL of XTT-labeling reagent (1 mg/mL XTT in DMEM) and 100 mL of electron coupling reagent (phenazine methosulfate, PMS). After 4 h of incubation, the optical density (OD) of sample was measured with an ELISA reader (Multiskan EX, Labsystems, MA, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

1.4 *Detection of cell cycle progression by flow cytometry*

HepG2 cells were cultured at 5×10^5 cells/mL in triplicate. After 48 h of incubation, the cells treated with or without the spider venom were analyzed. The cells were collected and suspended with 70% ethanol for 1 h at 4 °C, and then collected by centrifugation and resuspended in 0.4 mL of PBS containing 25 mL of 0.5% Triton X-100 and 10 mL of RNase (10 mg/mL) in water bath at 37 °C for 1 h. After staining with propidium iodide (PI) in the dark at room temperature for 30 min, the cell suspensions were filtered through a 40-mm mesh filter (BD Biosciences, NJ, USA) and analyzed by EPICS flow cytometer (Coulter Electronics, FL, USA). Data from 10 000 cells of each sample were collected for evaluation. The cell/organelle sizes and sub-G_1 peak analysis were evaluated with WinMDI version 2.5 software (TSRI Flow Cytometry). The cell cycle progression analysis was performed with MultiCycle AV software (Phoenix Flow Systems, San Diego, CA, USA).

1.5 *RT-PCR*

Total RNA was extracted by using TRIzol reagent (Gibco, Inc.). RNA quality and quantity were determined by ultraviolet (UV) spectrophotometry and 1.2% agarose gel electrophoresis. About 200 ng of total RNA was reversely transcribed using Super S with Platinum Taq kit (Invitrogen, Inc.) using the following primers: forward 5'-AGGATCATGTCAGAACCGGCTGG-3' and reverse 5'-CAGGATCCTGTGGGACGATTAGGAGG C-3' with the following conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 25 s, 56 °C for 15 s, and 72 °C for 40 s. PCR reaction was completed by incubation for 5 min at 72 °C. RT-PCR products were then separated on a 1.0% agarose gel, visualized under UV light and photographed. β-actin served as an internal control.

1.6 *Western blot*

HepG2 cells treated with different concentrations of the spider venom (0, 10, 20 and 40 μg/mL) were incubated respectively at 2.5×10^5 cells/well for 48 h, followed by a further cultivation for 48 h with 1 mL of serum-free RPMI 1640. Proteins of the cells were isolated by lysis buffer [100 mmol/L Tris-HCl, pH 6.8, 4% (*W/V*) SDS, 20% (*V/V*) glycerol, 200 mmol/L β-mercaptoethanol, PMSF 1 mmol/L, aprotinin 1 mg/mL, Sigma] and measured by using the Bradford assay with Bio-Photometer (BioPhotometer 6131 GB/HK, Eppendorf) at 595 nm. Equal amount of proteins in each sample was resolved in 10% SDS-PAGE and transferred onto nitrocellulose membranes. After washing in 10 mL/L fat-free milk, the membranes were incubated with the appropriate dilution of rabbit polyclonal p21 antibody and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody. Then they were incubated with a horseradish peroxidase-conjugated secondary

antibody. Proteins were detected by using the enhanced chemiluminescence (ECL) kit according to the manufacturer's protocol (Amersham, Buckinghamshire, UK).

1.7 *Statistical analysis*

Data were shown as mean±SD from three independent experiments. Statistically significant difference was identified by Student's *t*-test for paired data. $P<0.05$ was considered significant.

2 RESULTS

2.1 Cell growth inhibition assessed by colorimetric XTT assay

Concentration-dependent inhibition of HepG2 cells by the spider venom was observed. IC_{50} for 24 h is 12 μg/mL. When the concentrations ranged from 10 to 40 μg/mL, the inhibitory effect became obvious in a concentration-dependent manner (Table 1).

2.2 The spider venom induces G_2/M cell cycle arrest

To examine the mechanism of inhibitory effect of the spider venom on HepG2 cell proliferation, cell cycle progression was evaluated by using flow cytometry. As shown by the broadening of the pattern in Fig.1, cell cycle progression proceeded during the 48-hour incubation period. The spider venom caused an accumulation of cells at G_2/M phase. A dose-dependent effect on cell arrest at G_2/M phase was noted. It was found that the increment in G_2/M cell population was accompanied with a concomitant decrement of cell number at G_0/G_1 phase.

Table 1. Inhibition of cell proliferation by the spider venom

Spider venom (μg/mL)	*A*	Growth inhibitory rate (%)
PBS	0.246±0.003	0
DDP	0.035±0.004	90.27±5.49
40	0.087±0.003	84.41±4.73**
20	0.100±0.001	69.05±4.21*
10	0.132±0.003	46.14±3.82*

mean±SD. *$P<0.05$, **$P<0.01$ *vs* PBS.

2.3 Inhibitory effects of the spider venom on *p21* mRNA expression in HepG2 cells

To determine if the cell cycle arrest in HepG2 cells treated by the spider venom of 0, 10, 20 and 40 μg/mL was *p21*-dependent or not, we assessed the mRNA level of *p21* in HepG2 cells using RT-PCR assay. HepG2 cells were treated with the spider venom for 48 h. The results indicated that the expression of *p21* markedly increased after12 h of incubation, and was maintained at high concentration of the spider venom (Fig.2). The results showed that the activation of *p21* gene in HepG2 cells was apparent, suggesting that the spider venom possibly caused selective cell cycle arrest in HepG2 cells via activation of *p21*, which is postulated to associate with the *p21*-dependent pathway.

2.4 Effects of the spider venom on the activity of p21 protein

In order to elucidate the pathway leading to apoptosis, we examined the activation of p21 protein, which was reported to initiate apoptosis upon various stimuli. HepG2 cells treated with the spider venom (0, 10, 20 and 40 μg/mL) for 48 h were analyzed for the enzymatic activity by Western blot. p21 activity in HepG2 cells was increased after treatment for 48 h (Fig.3). The activity was enhanced significantly ($P<0.05$).

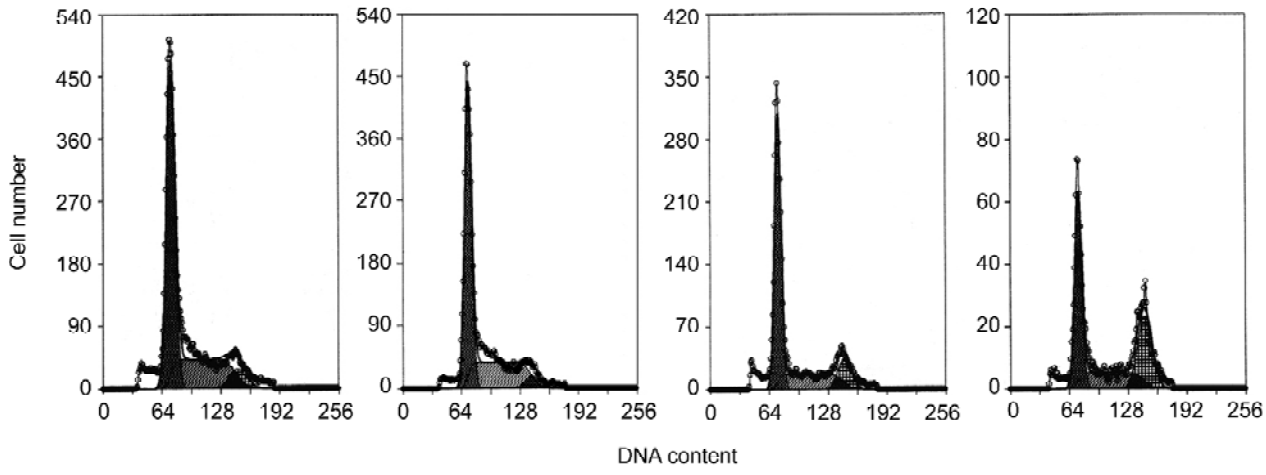

Fig.1. Effect of the spider venom on HepG2 cell cycle distribution.

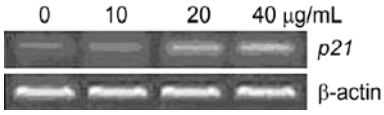

Fig.2. Expression of *p21* mRNA in HepG2 cells treated with different concentrations of the spider venom by reverse transcription-polymerase chain reaction analysis.

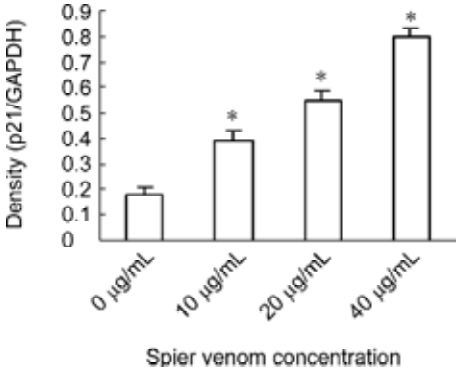

Fig.3. Western blot of p21 protein in HepG2 cells cultured for 48 h in the presence of 0, 10, 20 and 40 μg/mL of the spider venom. $^{*}P<0.05$ *vs* 0 μg/mL.

3 DISCUSSION

High-risk for primary hepatocellular carcinoma happens in China. Treatment for primary hepatocellular carcinoma is still difficult and depends on basic medical research. Recent evidence suggests that apoptosis of cells is closely related to the occurrence, progress and metastasis of tumors[6-8]. In this study, we demonstrate that the spider venom is very effective in inhibiting the proliferation of HepG2 cells.

Cells excluding vital dyes were considered to be viable. Our current results suggest that many of these cells are actually undergoing cell death. As shown in Table 1, the spider venom significantly induced inhibition of growth in a concentration-dependent manner in HepG2 cells after 24-hour treatment. The spider venom at the dose of 0, 10, 20 and 40 μg/mL significantly decreased cell proliferation measured by XTT assay in a dose- and time-dependent manner in HepG2 cells.

Some biological and biochemical effects of the spider venom were investigated in this study. To examine whether growth inhibition of HepG2 cells by the spider venom was a result of cell cycle arrest, the cell cycle treated with the spider venom was analyzed by flow cytometry. The inhibitory effect of the spider venom on growth of the cells may be due to G_2/M cell cycle arrest. The change of gene also showed an interesting pattern of the expression of positive regulators of cell cycle following treatment with the spider venom. It was then of interest to determine that the cell cycle in HepG2 cells could be inhibited by the spider venom.

Treatment strategies could therefore include not only up-regulation of gene and protein expressions but also targeting various steps in signaling between receptors and effector proteins and/or affecting expression or action of genes (proteins) differentially regulated[9-12]. p21 protein and *p21* gene were identified to be up-regulated in the spider venom-treated hepatoma cancer cells. Our results indicate that the accumulation of p21 protein following the spider venom treatment may be transcriptionally regulated, as both p21 protein and *p21* mRNA levels were increased following exposure to the spider venom (Fig.2 and 3). The increase in p21 protein might be related to cell cycle arrest.

It has been reported that the effect of cell cycle is associated with the up-regulation of downstream *p21* target gene. p21 protein is poised to play an essential role in growth arrest after DNA damage and contributes to the regulation of G_2/M transition[13,14]. Studies have shown that *p21* is necessary to maintain a G_2/M arrest following DNA damage. The mechanism of *p21*-dependent G_2/M arrest involves an initial inhibition of cyclin B1/cdc2 activity by *p21* gene levels[15-17]. In summary, this finding suggests that the elevation of *p21* expression may lead to cell cycle arrest through *p21* signal pathway.

The mechanisms regarding the inhibition of tumors have not been fully elucidated and might involve increase in *p21* gene and p21 protein expressions. Cyclin B1/cdc2 complex was known to be a key regulator of the transition from G_2 to M phase in the cell cycle[18-20], which might partly explain the dose-dependent induction of apoptosis in HepG2 cells by the spider venom. Our prolonged observation revealed that apoptosis did also occur with the spider venom at different concentrations. p21 overexpression accompanied with inhibition of cyclin B1/cdc2 complex has been linked to the G_2/M arrest in several cancer lines[21,22]. This might be explained by the fact that part of these apoptotic cells have derived from the sustained G_2/M arrest or HepG2 cells, as disclosed in our cell cycle analysis.

The spider venom increased the accumulation of *p21* in HepG2 cells, and further enhanced the level of *p21*, which could possibly associate with the arrest of G_2/M phase in the cell cycle. Therefore, the mechanism of chemo-sensitivity of HepG2 cells to the spider venom possibly dependends on *p21* pathway. Furthermore, our results also

demonstrate that the spider venom is a potent anti-proliferative agent against HepG2 cells, which exhibits a similar potency as the commercial oncostatic agent, DDP.

* * *

ACKNOWLEDGEMENT: We would like to thank Prof. LIU Jing-Ze for his technical and material assistance. We thank Paula E. Cushing and Dr. Gail Stratton (University of Mississippi) for helping us improve the manuscript.

REFERENCES

1 Okuda K. Hepatocellular carcinoma: recent progress. Hepatology 1992; 15(5): 948-963.

2 Zhu MS (朱明生), Li TH, Song DX. A species of genus macrothele (*Araneae*: *Hexathekidae*) from China. J Hebei Univ (河北大学学报) 2000; 20(4): 358-361 (Chinese, English abstract).

3 Zeng XZ (曾雄志), Liang SP. Purification and preliminary toxin research of raventoxin-II, a neurotoxic peptide from the venom of the spider *Macrothele raveni*. Life Sci Res (生命科学研究) 2001; 5(3): 217-220 (Chinese, English abstract).

4 Zeng XZ, Xiao QB, Liang SP. Purification and characterization of raventoxin-I and raventoxin-III, two neurotoxic peptides from the venom of the spider *Macrothele raveni*. Toxicon 2003; 41(6): 651-656.

5 Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 1991; 142(2): 257-265.

6 Hashemi M, Karami-Tehrani F, Ghavami S, Maddika S, Los M. Adenosine and deoxyadenosine induces apoptosis in oestrogen receptor-positive and -negative human breast cancer cells via the intrinsic pathway. Cell Prolif 2005; 38(5): 269-285.

7 Duann P, Ho TY, Desai BD, Kapoian T, Cowen DS, Lianos EA. Mesangial cell apoptosis induced by stimulation of the adenosine A3 receptor: signaling and apoptotic events. J Investig Med 2005; 53(1): 37-43.

8 Gorczyca W, Bigman K, Mittelman A, Ahmed T, Gong J, Melamed MR, Darzynkiewic Z. Induction of DNA strand breaks associated with apoptosis during treatment of leukemia. Leukemia 1993; 7(5): 659-670.

9 Liu QS, Zhang RH, Chu YW, Xiong SD. Differential susceptibility of native versus cloned $CD4^+$ T cells to antigen-specific and MHC-restricted anergy induction. Acta Physiol Sin (生理学报) 2003; 55(6): 633-640.

10 Liu PC (刘鹏翀), Lu SD, Huang YL, Sun FY. The expression of nestin in ischemia-injured brain of adult rat. Acta Physiol Sin (生理学报) 2002; 54 (4): 294-299 (Chinese, English abstract).

11 Cao CM (曹春梅), Xia Q, Ye ZG, Zhang X. Effect of interleukin-2 on intracellular calcium transients in rat ventricular myocytes. Acta Physiol Sin (生理学报) 2001; 53(6): 425-430 (Chinese, English abstract).

12. Zang WJ, Chen LN, Yu XJ. Progress in study of vagal control of cardiac ventricles. Acta Physiol Sin (生理学报) 2005; 57(6): 659-672.

13. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 2002; 1(8): 639-649.

14 Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown PJ, Sedivy JM, Kinzler K W, Vogelstein B. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science 1998; 282 (5393): 1497-1501.

15 Fletcher L, Cheng Y, Muschel RJ. Abolishment of the Tyr-15 inhibitory phosphorylation site on cdc2 reduces the radiation-induced G_2 delay, revealing a potential checkpoint in early. Cancer Res 2002; 62(1): 241-250.

16 Fotedar R, Bendjennat M, Fotedar A. Role of the $p21^{WAF1}$ in the cellular response to UV. Cell Cycle 2004; 3(2): 134-137.

17 Stellrecht CM, Rodriguez CO Jr, Ayres M, Gandhi V. RNA directed actions of 8-chloro-adenosine in multiple myeloma cells. Cancer Res 2003; 63(22): 7968-7974.

18 Choi YH, Lee WH, Park KY, Zhang L. p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G_2/M arrest by the isoflavone genistein in human prostate carcinoma cells. Jpn J Cancer Res 2000; 91(2): 164-173.

19 Pumford SL, Morton MM, Turkes A, Griffiths K. Determination of the isoflavonoids genistein and daidzein in biological samples by gas chromatography-mass spectrometry. Ann Clin Biochem 2002; 39(3): 281-292.

20 Niculescu AB, Chen X, Smeets M, Hengst L, Prives C, Reed SI. Effects of $p21^{(Cip1/Waf1)}$ at both the G_1/S and G_2/M cell cycle transitions: pRb is critical determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol 1998; 18 (1): 629-643.

21 Chan TA, Hermeking H, Lenganer C, Kinzler KW, Vogelstein B. 14-3-3s is required to prevent mitotic catastrophe after DNA damage. Nature 1999; 401(6753): 616-620.

22 Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994; 266(5192): 1821-1828.