Effects of Seaweed Pigment Glycoprotein on Caspase-3 and Bax Protein Expression of Hepatoma Cells

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ABSTRACT Objective: To investigate the effect of seaweed pigment glycoprotein (SPG) on the expression of Caspase-3 and Bax protein in hepatoma cells. Methods: Fifty mice inoculated with hepatoma H_{22} cells were divided into five groups, 10 mice per group. The high, medium and low SPG group were administered by gastric perfusion with 100, 50 and 10 mg/kg/day SPG, respectively. The tumor control group was given the same amount of saline daily. The cyclophosphamide group was given 20 mg/kg cyclophosphamide by intraperitoneal injection every other day. The proliferation activity of hepatoma cells was determined by MTT assay. The expression of Caspase-3 and Bax protein were detected by immunohistochemistry method. Results: The expression of Caspase-3 and Bax protein in high-dose group were 40.20 % and 38.10 %, respectively, while those in the tumor control group were 5.00 % and 4.68 %. The differences between two groups were significant ($p<0.05$). The proliferation activity of high-dose group and tumor control group were 0.711± 0.028 and 1.135± 0.032.The difference was significant ($p<0.05$). Conclusion: SPG can promote the expression of Caspase-3 and Bax protein, and induce the apoptosis of hepatoma cells.

Key words: Seaweed pigment glycoprotein; Hepatoma cell; Proliferation; Caspase-3; Bax

Chinese Library Classification (CLC): Q95-3, R114 Document code: A

Article ID: 1673-6273 (2011) 09-1771-04

Introduction

Seaweed pigment glycoprotein, extracted from Gracilaria lemaneiformis, is a natural compound composed of phycobiliprotein and sulfated polysaccharide. Studies have shown that phycobiliprotein was a light-harvesting phycobiliprotein which was special in marine algae [1] and have anti-tumor, anti-oxidation and anti-mutagenic effects[2,3]. However, few reports have investigated the effects of a whole natural compound composed of phycobiliprotein and sulfated polysaccharide. This study examined the effects of seaweed pigment glycoprotein on the expression of caspase-3 and Bax protein of hepatoma cells.

1 Materials and methods

1.1 Materials

1.1.1 Animals The ascitic-type hepatoma H_{22} donor mice was provided by the Chinese Academy of Medical Sciences, A total of 50 healthy Kunming mice (25M, 25F) weighing 18~22 g, were provided by Shandong Lukang Laboratory Animal Center (SCXK-LU20080002).

1.1.2 Samples SPG Samples SPG was extracted in our laboratory and the purity was greater than 90 %.

1.1.3 Reagents and Equipments Cyclophosphamide (CTX) were purchased from Jiangsu Hengrui Medicine Co., Ltd. RPMI 1640 medium, calf serum, Thiazolyl Blue Tetrazolium Bromide (MTT) and typan blue reagent were purchased from Sigma Chemical Corporation. Caspase-3, Bax immunohistochemisty detection kits and DAB were purchased from Wuhan Boster Corp. Light microscope and phytomicrographic camera PM-CBK-G were products of Olympus, Japan. Microplate reader (Bio-Rad model 550) was bought from Sweden.

1.2 Methods

Fifty Kunming mice were randomly divided into five groups, 10 mice per group. Each mouse was vaccinated 0.2 mL suspension with the concentration of 2×10^6 /mL of H_{22} tumor cells in right fore axillary subcutaneously. 24 hours later, the mice were intervened according to the weight. Mice in high, medium and low SPG groups were given 100, 50, 10 mg/kg·d of SPG by gastric perfusion, respectively. CTX group was given 20 mg/kg·d CTX by intraperitoneal injection. Tumor control group was given the same amount of saline. Two weeks later, all mice were sacrificed and tumors were separated.

1.2.1 Cell proliferation Cell suspension was made and adjusted cell density was adjusted to 1×10^6 cells/mL. 100 μL cell suspension was added into the wells of 96-well plates and cultured for 16 h at 37℃ in humidified atmosphere containing 5 % CO_2. Each mouse had three parallel wells. After incubation, cell proliferation activities were subsequently measured using MTT assay. Briefly after MTT reagent was added to each well and color developed, the absorbance of each sample was measured using a microplate reader, and results were expressed with average optical density (A).

1.2.2 The expression of Caspase-3 protein Caspase-3 protein was detected by immunohistochemical method. The paraffin embedded samples of tumor tissue were sliced and dewaxed by normal methods. Then tissue-section were hydrated and rinsed in

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(Received: 2011-01-05 Accepted:2011-01-31)

PBS. After subsequent inactivation of intrinsic enzymes with 3 % H_2O_2, exposure of antigen sites with 3 % citric acid diluted pepsin, pre-hybridization and coloration, the expression of Caspase-3 protein was observed under light microscopy (× 400). For each mice, 5000 cells were detected and the number of Caspase-3 positive cells was counted.

1.2.3 The expression of Bax protein After the histological section mentioned above, all experiment procedures were conducted according to the manufacturer's instructions. The expression of Bax protein was observed under light microscopy (× 400).

1.3 Statistical Analysis

One-way analysis of variance was used to the categorical variable data and Chi-square test was used to the numerical variable data. All analysis was performed using SPSS 17.0.

2 Results

2.1 Cell proliferation

The proliferation activity levels of high dose group and tumor control group were 0.711± 0.028 and 1.135± 0.032, respectively. There was significant difference between the two groups(P<0.05). The results of cell proliferation are shown in Table 1.

2.2 Caspase−3 protein expression

Caspase-3 expressed as yellow-brown granules in nuclei and cytoplasm (× 400). The Caspase-3 expression in the high dose group was higher than that the tumor control group (Fig. 1). The caspase-3 expression levels in the high dose group and the tumor control group were 40.20 % and 5.00 %, respectively, which were significantly different (p <0.05). The Caspase-3 expression levels in each group were shown in Table 2.

Table 1 The proliferation activity levels of the mice hepatoma cells in each group ($\bar{x}$± s, n=10)

Group	A value	IR (%)
Tumor control	1.135± 0.032	—
Low dose	1.055± 0.069[b]	7.05
Medium dose	0.825± 0.020[a,b]	27.31
High dose	0.711± 0.028[a]	37.36
CTX	0.679± 0.056[a]	40.18

a : p＜0.05 compared with tumor control; b : p＜0.05 compared with high dose

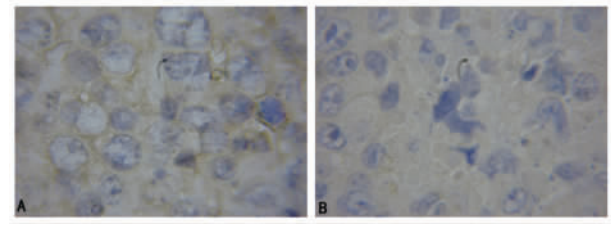

Fig.1 The expression of Caspase-3 in hepatoma cells (DAB× 400) : A High-dose SPG group; B Tumor control group

Table 2 The protein expression of Caspase-3 and Bax in each group

Group	n	Caspase-3		Bax	
		Positive cells (n)	Positive rate (%)	Positive cells (n)	Positive rate (%)
Tumor control	5000	250	5.00	234	4.68
Low dose	5000	295	5.90[b]	259	5.18[b]
Medium dose	5000	874	17.50[a,b]	897	17.90[a,b]
High dose	5000	2009	40.20[a]	1904	38.10[a]
CTX	5000	2053	41.10[a]	2021	40.40[a]

n=number of observed cells; a : p＜0.05 compared with tumor control; b : p＜0.05 compared with high dose

2.3 Bax protein expression

Bax expressed as yellow-brown granules in nuclei and cytoplasm when observed under microscope (× 400). Compared with control group, there were more cells with yellow-brown granules and the color was lighter in the high dose group, indicating a stronger expression of Bax (Fig. 2). The Bax expression levels in the high dose group and the tumor control group were 38.1 % and 4.68 %, respectively, which were significantly different (P<0.05). The Bax expression levels in each group were shown in Table 2.

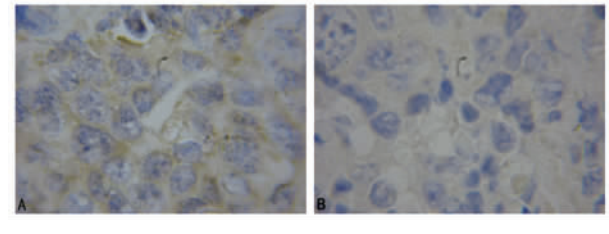

Fig.2 The protein expression of Bax in hepatoma cells (DAB× 400): A High-dose SPG group; B Tumor control group

3 Discussion

Several factors contribute to apoptosis, but the key elements are categorized into two main families of proteins including caspase enzymes and Bcl-2 family[4,5].The caspases are a family of intracellular cysteineproteases specificity targeted at aspartic acid residues [6]. Caspases are cystein proteases that play a key role in

the execution phase of apoptosis[7]. The activation of caspase is the key event during the induction of apoptosis [8]. Caspase-3, a member of the family of caspases, extensively studied as "the executor of apoptosis", plays a crucial role in cell death [9]. Apoptosis mediated by caspase-3 occurs in many cancer cells[10]. Among all downstream executioner caspases, caspase-3 is the most frequently reported effective caspase that induces apoptosis in various modes of cell deaths [11,12]. Following the activation of the apoptosis process by upstream caspases, Caspases-3 will cleave a variety of substrates, thus caspase-3 is considered to constitute a general terminal mediator of apoptosis [13,14]. When cells are damaged, activation of caspase-3 enhances. In this study, the caspase-3 expression of the mice in high and middle dose group was obviously higher compared with that of the tumor control group. The caspase-3 expression tended to rise with the increasing of SPG dose. It indicated that SPG promoted the caspase-3 expression and then induced apoptosis of hepatoma cells. This result is in agreement with the reports of Zhang YY on the anti-mutagenic and anti-tumor activity of phycobiliproteins from Gracilaria Lemaneiformis[2]。

Bcl-2 family is a set of cytoplasmic protein members that regulate apoptosis. The two main groups of this family are Bcl-2 and Bax proteins [15,16]. Bax is related with cell death [17]. Studies have shown that the expression of Bax induced apoptosis with apoptosis-related morphological changes, caspase activation with subsequent substrate proteolysis [18]. Bax protein plays a critical role in inducing apoptosis in tumor cells. In this study，the Bax expression of mice in the high and middle dose groups were significantly higher than that of the tumor control group. With the increasing of SPG, the Bax expression is elevated. This result is in accordance with the expression of caspase-3.

Cell proliferation is a sensitive and precise index for evaluating cell activity. MTT assay has been proved to be a very sensitive and rapid method for the quantitative cytotoxicity evaluation of biomaterials and is now routinely used in laboratories [19,20]. In this study, proliferation levels of the high SPG dose group significantly decreased compared with the middle, low SPG dose and tumor control groups, which indicated that seaweed pigment glycoprotein could significantly inhibit the proliferation activity of the H_{22} hepatoma cells. This data is supported by the result of the expression of caspase-3 and Bax.

In summary, this study showed seaweed pigment glycoprotein could significantly promote the expression of caspase-3 and Bax, inhibit the proliferative activity and induce apoptosis of H_{22} hepatoma cells.

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海藻色素糖蛋白对肝癌细胞 Caspase-3 和 Bax 蛋白表达的影响

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摘要 目的:研究麒麟菜海藻色素糖蛋白(SPG)对肝癌细胞 Caspase-3 和 Bax 蛋白表达的影响。方法:将 50 只皮下接种 H22 肝癌细胞株的小鼠随机分为 5 组,每组 10 只。高、中、低剂量组分别每天经口灌胃给予 100、50、10 mg/kg 的 SPG,肿瘤对照组灌胃生理盐水,连续 10d。环磷酰胺组隔天腹腔注射环磷酰胺 20 mg/kg.bw。取肝癌组织用 MTT 法测定各组肝癌细胞增殖活性,免疫组化法检测各组肝癌组织 Caspase-3 和 Bax 蛋白表达水平。结果:高剂量组 Caspase-3 和 Bax 蛋白表达率分别为 40.20 %和 38.10 %,而肿瘤对照组分别为 5.00 %和 4.68 %,差异均有显著性(P＜0.05)。高剂量 SPG 组和肿瘤对照组的肝癌细胞增殖活性分别为 0.711± 0.028 和 1.135± 0.032,差别有显著性(P＜0.05)。结论:SPG 可促进肝癌细胞 Caspase-3 和 Bax 蛋白表达,诱发肝癌细胞凋亡。

关键词:海藻色素糖蛋白;肝癌细胞;增殖活性;Caspase-3 蛋白;Bax 蛋白

中图分类号:Q95-3, R114 **文献标识码**:A **文章编号**:1673-6273(2011)09-1771-04

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(**收稿日期**:2011-01-05 **接受日期**:2011-01-31)