RESEARCH ARTICLE

Immune and Anti-oxidant Functions of Ethanol Extracts of *Scutellaria baicalensis* Georgi in Mice Bearing U14 Cervical Cancers

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Abstract

Background: The objective was to study the effect of *Scutellaria baicalensis* Georgi ethanol extracts (SBGE) on immune and anti-oxidant function in U14 tumor-bearing mice. Materials and Methods: U14 tumor-bearing mice were randomly divided into eight groups: a control group, a cyclophosphamide (CTX) group, three dose groups of SBGEI (high, medium, low), and three dose groups of SBGEII (high, medium, low). After two weeks, the thymus and spleen weight indices of mice bearing U14 cervical cancer were calculated. Enzyme linked immunosorbent assays (ELISA) was used to determine the levels of serum IL-2, TNF-α, IL-8, and PCNA. MDA activity and SOD activity in plasma were measured with detection kits. Results: In the SBGE groups, thymus weight and spleen weight indices of U14 tumor-bearing mice were significantly higher than in the control group or CTX group ($p<0.05$). Compared to control group, the levels of serum IL-2 and TNF-α in U14 tumor-bearing mice increased significantly, whereas the contents of serum IL-8 and PCNA decreased ($p<0.05$). The activity of SOD increased with the growing dose of SBGE, while the activity of MDA decreased significantly in the higher-dose groups of SBGE. Conclusions: These findings suggested that SBGE, especially at high dose, 1000 mg/kg, showed significant immune and anti-oxidant effects infU14 tumor-bearing mice, which might be the mechanisms of SBGE inhibition of tumor growth.

Keywords: SBGE - U14 cervical cancer - immune - anti-oxidant - functions

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Introduction

Scutellaria baicalensis Georgi (SBG) is a traditional Chinese herbal medicine used to clear fever, release fire, neutralize poison, stop bleeding and prevent miscarriage in the form of decoction or extracts. It is confirmed that the active components of the root of SBG are flavonoids which have anti-inflammatory and anti-allergic effects, anti-oxidant, anti-thrombotic and vasoprotective properties (Su et al., 2008; Kesarkar et al., 2009). Previous studies have also reported that ethanol extract of SBG could prevent oxidative damage and show anti-oxidation, anti-inflammation and induction cell apoptosis effects (Jeong et al., 2011; Lu et al., 2011; Lin et al., 2013). However, the effects of SBG on immune and anti-oxidant function of mice bearing tumors, especially cervical cancer, are relatively unknown.

Cervical cancer which is mainly infected by high risk human papilloma virus (HR-HPV, HPV16 and HPV 18 were the commonest genotypes) is one of the most common cancer affecting women worldwide (Bosch et al., 1995; Wang et al., 2013). In many developing countries, it is the leading cause of cancer-related death, moreover, cervical cancer affects individuals at a younger age than other cancers do; almost 50% of cases are detected before the age of 35 (Oaknin et al., 2012). Therefore, it will have important significance to prevent the occurrence of cervical cancer in the early lesions.

In our previous study, the results have showed that *Scutellaria baicalensis* Georgi ethanol extracts (SBGE) could inhibit the tumor growth with the highest inhibition rate of 59.86% and induce the apoptosis of tumor cells (Peng et al., 2012). In order to explore the anti-tumor effects of SBGE and its influences to the immune and anti-oxidant function of the tumor-bearing mice, the further research was carried out. In the present study, U14 tumor-bearing mice model was established by the inoculation of cancer cell suspension subcutaneously in the mouse's left anterior limb. Different groups of mice were given corresponding medicines for 15 days, compared to other group, we examined the effect of SBGE on immune and anti-oxidant function of U14 tumor-bearing mice to evaluate its potential mechanism in the prevention and treatment of cervical cancer.

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Materials and Methods

Agents

SBG was purchased from Min Le Pharmacy (Qinhuangdao, China). CTX was provided by Pude Pharmacy Inc. (Shanxi, China), with Lot number 1008421. Mouse interleukin-2 enzyme-linked immunoassay kit (IL-2/CXCL8 ELISA Kit), mouse tumor necrosis factor-α enzyme-linked immunoassay kit(TNF-Alpha ELISA Kit), mouse interleukin-8 enzyme-linked immunoassay kit (IL-8/CXCL8 ELISA Kit) and mouse anti-proliferating cell nuclear antigen antibody enzyme-linked immunoassay assay kit (PCNA ELISA Kit) were supplied by R&D Company (America). Commercial kits to measure superoxide dismutase (SOD) and malondialdehyde (MDA) activity were from Beyotime Institute of Biotechnology in China. All other chemicals used were of analytical grade.

Animals

Healthy Kunming female mice at 6 weeks of age (weighing 18-22 g) were provided by the Experimental Animal Center of Chinese Military Academy of Medical Sciences, with Lot Number SCXK-(Army) 2007-004. Mice were housed in a clean animal feeding room with food and water ad libitum. The room was maintained appropriate temperature (20±2°C) and humidity (50-60%), and provided 8 to 10 h illumination every day.

Cell line

Mouse U14 cervical cancer cell line was obtained from Tumor Hospital of the Chinese Academy of Medical Sciences.

Preparation of SBG ethanol extracts

The powder of SBG (500g) was extracted three times for 3 h each with 90% ethanol at 80°C. The extracts were filtered, combined, concentrated using a rotary evaporator, and got through the silica gel column chromatography, yielding the 30% ethanol extract of SBG based on the dry weight of SBG root. 50% ethanol extract of SBG were prepared according to the same procedure but with 50% ethanol. After drying by the vacuum freeze drier, the resulting drugs was collected and weighed. The two kinds of ethanol extracts were named as SBGEI and SBGEII respectively.

Experimental design

Under sterile conditions, the ascitic cells taken from mice that had tumors U14 for 7 d were diluted to a density of 1.60×10^6/ml with physiological saline. Then 0.2 ml U14 cells were inoculated subcutaneously in the left axilla of per mouse. The success rate of inoculation was 100%.

After tumor inoculation, mice were randomly divided into 8 groups (10 mice in each group). In the control group, each mouse was fed 0.2 ml distilled water daily, while in the CTX group each mouse was injected of 0.2 ml CTX (25mg/kg) daily in abdomen. SBGEI, II were divided separately into three groups with different doses: high dose ethanol extract group (1000 mg/kg), medium dose ethanol extract group (500 mg/kg), low dose ethanol extract group (250 mg/kg). All the treatment groups were continuously given corresponding medicines (0.2 ml) for 14 days.

Determination of thymus and spleen weight index in mice bearing tumor

The mice of all groups were euthanized by cervical dislocation at the 15th day, the thymus and spleen were taken out and weighed, the weight indexes of thymus and spleen were calculated by the follow formulas:

Thymus weight index (mg/10g)=the average thymus weight (mg)/the average body weight (g) ×10

Spleen weight index (mg/10g)=the average spleen weight (mg)/the average body weight (g) ×10

Determination of immune indexes

At the 15th day, mice of all groups were weighed and extracted the eyeball blood. After 20 minutes' standing, the anticoagulated blood samples were centrifuged for 20min at 3000 rpm. The levels of IL-2, TNF-α, IL-8 and PCNA in the supernatant were determined by using enzyme-linked immunosorbent assay (ELISA) kits.

Determination of anti-oxidant indexes

By the end of the experiment on day 15, all mice eyeball blood collected into the anticoagulant tube was centrifuged at 2000 rpm for 10 min at 4°C, then the supernatant was removed to another centrifuge tube and diluted with physiological saline. The SOD activity and MDA content in the supernatant were measured by detection kits.

Statistical analysis

Statistical analyses were performed with the SPSS package software version 13.0. All data are expressed as the mean±S.D., differences between groups were tested by unpaired Student's t-tests. All $p<0.05$ were considered statistically significant.

Results

Effects of SBGE on thymus and spleen weight index of mice bearing U14 cervical cancer

Comparing with the negative control group, after treatment by SBGE, the thymus and spleen weight index of mice bearing U14 cervical cancer have different degrees of improvement ($p<0.05$, $p<0.01$), while the thymus and spleen weight index were declined in the CTX group ($p<0.05$). The results suggested that CTX could not improve immune function, whereas SBGE could improve the immunity of mice bearing U14 cervical cancer (Table 1).

Effects of SBGE on the activity of immune prevention of mice bearing U14 cervical cancer

Comparing with the control group, the activity of immune defenses of mice bearing U14 cervical cancer in the CTX group was strengthened ($p<0.05$). Mice treated by SBGE in each group showed significant improvement in the levels of serum IL-2 and TNF-α with the increasing of SBGE dose ($p<0.05$). And the expression levels of

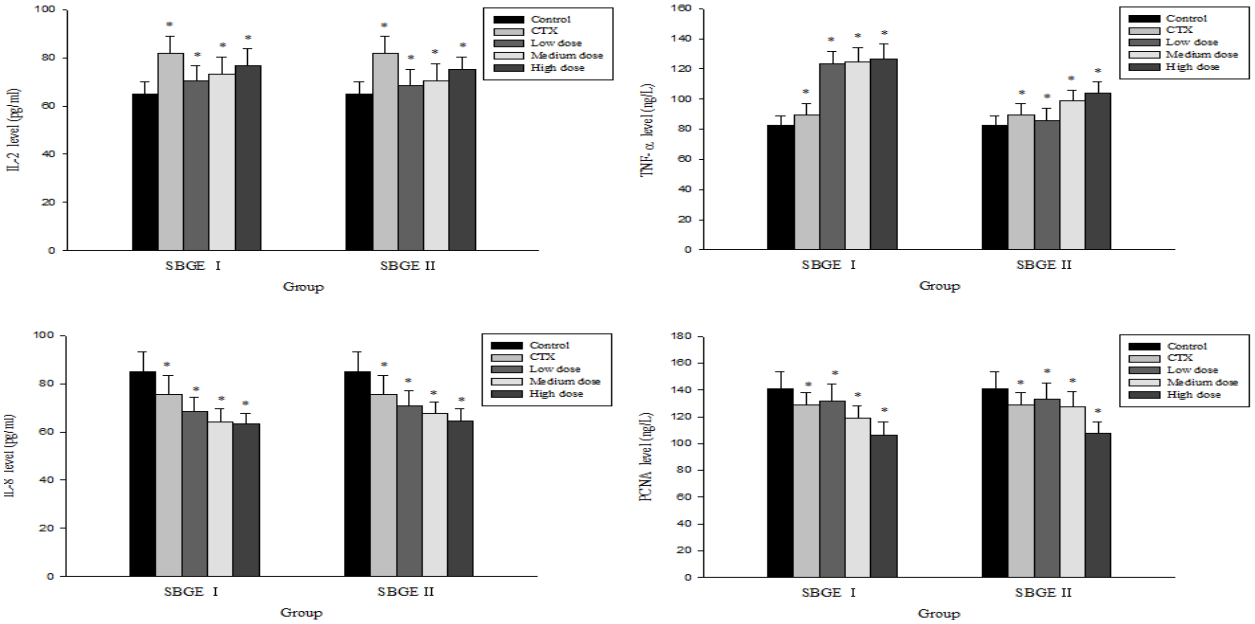

Figure 1. Effects of SBGE on the Expression Levels of IL-2, TNF-α, IL-8, PCNA in Mice Bearing U14 cervical cancer *p<0.05 as Compared with Model Control Group

Table 1. Effects of SBGE on Thymus and Spleen Weight Index of Mice Bearing U14 Cervical Cancer

Group	Treatment (mg/kg/day)	Thymus index (mg/10g)	Spleen index (mg/10g)
Control	—	9.129±0.814	50.023±3.757
CTX	25	4.865±0.387*	38.425±2.385*
High dose of SBGEI	1000	17.843±1.535*	76.926±4.835*
Medium dose of SBGEI	500	16.145±1.398*	71.627±3.452*
Low dose of SBGE I	250	15.166±1.268*	65.783±5.337*
High dose of SBGE II	1000	16.253±1.761*	77. 234±4.314*
Medium dose of SBGE II	500	14.895±1.538*	68.162±4.586*
Low dose of SBGE II	250	11.738±1.565#	64.847±5.450*

*p<0.05, #p>0.05 as compared with control group

Table 2. Effects of SBGE on the Activity of Anti-Oxidant of Mice Bearing U14 Cervical Cancer

Group	Treatment (mg/kg/day)	SOD (U/ml)	MDA (nmol/ ml)
Control	—	221.135±14.134	6.360 ±0.441
CTX	25	301.651±17.153*	3.771±0.316*
High dose of SBGE I	1000	293.440±17.524*	2.627±0.248*
Medium dose of SBGE I	500	259.083±18.272*	3.131±0.417*
Low dose of SBGEI	250	236.457±16.338*	3.869±0.284*
High dose of SBGE II	1000	305.432±18.157*	2.386±0.325*
Medium dose of SBGE II	500	264.847±15.348*	3.071±0.409*
Low dose of SBGE II	250	241.571±17.365*	3.682±0.359*

*p<0.05 as compared with control group

serum IL-8 and PCNA were lower than those in model group (p<0.05). The experimental results indicated the SBGE could improve the immune defense functions of mice bearing U14 cervical cancer (Figure1).

Effects of SBGE on the activity of anti-oxidant of mice bearing U14 cervical cancer

After mice bearing U14 cervical cancer were treated with SBGE, the plasma SOD contents were increased significantly, while the plasma MDA activities were decreased (p<0.05) in the SBG group, as compared with that in the control group. The results showed that SBGE had notable anti-lipid peroxidation effect on mice bearing U14 cervical cancer, probably due to enhancing the anti-oxidant effect and inhibiting the generation of oxygen free radicals (Table 2).

Discussion

The immune system plays a major role in the defense against cancer when normal cells begin to grow out of control because of mutant DNA sequences. Therefore, strengthening the immune system or enhancing the body's immune function to fight cancer is an appealing prospect. CTX is one of the most clinically important drugs for malignant tumors, which has broad-spectrum anti-cancer and immune suppression effects. In our study, CTX was used as the positive control group to evaluate the effect of SBGE on the immune function of tumor-bearing mice. These tests showed that the thymus of mice transplanted U14 cervical cancer began to shrink. While after treatment with SBGE of two weeks, compared with the CTX group, the thymus indexes increased markedly (p<0.05, p<0.01), indicating that SBGE may have activities of delaying thymus involution, preventing lymphocyte damage and promoting thymus lymphocyte proliferation. Meanwhile, the spleen weight of tumor-bearing mice decreased significantly with the growth of the tumor. The spleen indexes of the SBGE groups were increased dramatically in contrast to the control group (p<0.05), while the spleen weight indexes of CTX group were decreased significantly (p<0.05). This result showed that SBGE might adjust immunity through encouraging primitive cells' differentiation into T cells in the spleen hematopoietic tissue, while CTX could not enhance the immunity of mice. This study showed that SBGE significantly improved the thymus and spleen weight index of tumor-bearing mouse, enhanced specific and non-specific immune responses, which played an important role in the inhibition of tumor growth.

Some studies revealed that some cytokines produced

by cells of the innate defense system have direct or indirect anti-tumor or immune-enhancing effects (Belardelli et al., 2002). As a cytokine that plays an essential role in cell-mediated immunity, IL-2 is involved in T cell activation and differentiation, can strengthen cell anti-reactive and stimulate B cell proliferation and immunoglobulin secretion (Mingari et al., 1984). In addition, IL-2 is also a growth factor for natural killer (NK) cells. In some studies IL-2 has been shown to have powerful anti-tumor effects and has been used for the treatment of melanoma and renal cell carcinoma in the clinic (Overwijk et al., 2000). TNF-α is a multifunctional cytokine that possesses anti-tumor activity including destruction of the tumor endothelial cells, increasing vascular permeability (van Etten et al., 2003), inhibition of tumor angiogenesis mediated by generation of angiostatin (Mauceri et al., 2002). TNF-α can not only inhibit the growth of tumor cells but also kill tumor cells directly through cytolysis. In our study, SBGE significantly enhanced the expression levels of IL-2 and TNF-α in tumor-bearing mice, which could be the possible mechanism of its anti-tumor effect.

It is clear that IL-8, a proinflammatory CXC chemokine, has angiogenic activity. Some studies revealed that IL-8 played an important role in stimulating tumor cell proliferation, migration, and invasion (Luppi et al., 2007; Yao et al., 2007; Waugh et al., 2008). Therefore, inhibiting the expression of IL-8 may be a strategy for cancer therapy. Proliferating cell nuclear antigen (PCNA), also known as cycle antigen, is the necessary material for DNA replication, whose content reflects the degree of cell proliferation. In recent years, increasing studies demonstrated that PCNA may be not a valuable marker to predict the progression of cervical cancer, but it is closely associated with high-risk human papillomavirus (HPV) and progression of cervical intraepithelial neoplasia (CIN), so the index of PCNA is potentially helpful for predicting the prognosis in cervical cancer (Zhang et al., 1999; Wang et al., 2004; Branca et al., 2007). Our studies showed that after treatment with SBGE, serum IL-8 and PCNA of U14 tumor-bearing mice decreased ($p<0.05$), implying that SBGE may inhibit tumor angiogenesis, invasion and metastasis by reducing the content of IL-8 and PCNA, which also may be an effect mechanism of SBGE on cervical cancer.

It is well known that oxygen-free radicals, also known as reactive oxygen species (ROS), which is playing a key role in oxidative damage accumulating during the life cycle, thus induce the development of age-dependent diseases such as cancer (Valko et al., 2006). SOD is an essential enzyme that scavenges oxygen-free radicals and protects cells from oxidative damage. MDA, the end product of the lipid peroxidation caused by attacking on biofilm polyunsaturated fatty acids, is known as a biological marker of free radicals in the body.

Our study results showed that compared with control group, the activities of plasma SOD increased ($p<0.05$), while the contents of plasma MDA decreased ($p<0.05$) significantly in the SBGE group. We deduced that SBGE may enhance the level of SOD to eliminate excessive free radicals, which resulted in reducing the lipid peroxidation, suggesting that SBGE exerts an anti-cervical cancer effect probably through enhancing the anti-oxidant activity of tumor-bearing mice.

In conclusion, our study demonstrated that SBGE may effectively enhance the immune function and the anti-oxidant capability of tumor-bearing mice, thus delaying the process of tumor growth and development, and inhibiting the proliferation of cervical cancer.

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