

Experimental Studies of the Therapeutic Effect of *Gypsophila Oldhamiana* Gypsogenin on Lewis Lung Cancer in Mice

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OBJECTIVE To observe the inhibitory effect of *Gypsophila oldhamiana* gypsogenin (GOG) on Lewis lung cancer growth, and to investigate the mechanism of its anti-tumor effect.

METHODS A mouse model bearing Lewis lung cancer was used. The 5 experimental groups were divided into a positive control (cis-diaminedichloroplatinum), a negative control, and high, medium and low-GOG dosage groups. The inhibitory action of GOG administration on the lung cancer was observed. After GOG treatment, the lungs were taken out and a lung coefficient computed. The expressions of VEGF, Bcl-2, Bax and P53 in the cancers were determined using immunohistochemical staining.

RESULTS The tumor weight of the mice given various doses of GOG was significantly lower compared to the negative-control group ($P < 0.01$), and the lung coefficient of the groups given high and low GOG doses was significantly lower compared to the negative-control group ($P < 0.01$). Immunohistochemical results were shown as follows: *i*) The VEGF and Bcl-2 expressions in the GOG groups were significantly lower than that of the negative-control group ($P < 0.05$); *ii*) Bax expression in the groups treated with high and medium GOG doses was significantly higher compared to the negative-control group ($P < 0.05$); *iii*) The mutant P53 expression in the GOG-treated groups was significantly lower compared to the negative-control group ($P < 0.05$).

CONCLUSION GOG can inhibit the growth and metastasis of Lewis lung cancer, and may exert its mechanism of tumor control by inhibition of tumor angiogenesis and induction of apoptosis.

KEY WORDS: *Gypsophila oldhamiana* gypsogenin, Lewis lung cancer, tumor growth, tumor metastasis.

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Introduction

Gypsophila oldhamiana gypsogenin (GOG) is a triterpenoid saponin separated and extracted from the root of *Gypsophila oldhamiana* Miq (GOM or tumble-weed), a Caryophyllaceae plant. This root, which is called the starwort root, has an antipyretic effect. It has been usually used as a folk medicine to treat symptoms or diseases such as weakness and tiredness, chronic malaria with yin asthenia and infantile malnutrition with fever, caused by febrile and wasting diseases, such as rheumatic disease and lung tuberculosis etc. Yang et al.^[1] have obtained gypsogenin, a sterol and fatty acid, etc. from this root. Findings from the Chinese literature showed that gypsogenin from dioecism *Momordica* root has an obvious inhibitory effect in has on L1210, CCER-CEM and LS174T cancer cells^[2]. In our study, GOG was obtained following separation and purification of a methanol-soluble fraction of the GOG root. The aim of this study was to search for anti-lung cancer effects of GOG, and to preliminarily investigate its mode of action.

Materials and Methods

Drug and main reagents

GOG is a white powder that was provided by the Naturally Occurring Drug Laboratory of our institute. GOG was dissolved in 0.9% NaCl, sterilized by filtration and adjusted to various concentrations for injection. The cis-diaminedichloroplatinum (CDDP) for injection was produced by the Qilu Pharmaceutical Co., Ltd. A Polylysine, immunohistochemical kit and DAB developer were procured from the Beijing Zhongshan Golden Bridge Biotechnology Co., China. Mouse-antihuman VEGF monoclonal antibodies and rabbit-antimouse Bax polyclonal antibodies were purchased from the Santa Cruz Co., USA. Rabbit-antihuman P53 and Bcl-2 polyclonal antibodies were obtained from the Wuhan Boster Bioengineering Co., Ltd., China.

Animals

A total of 71 healthy, male inbred C57BL/6J mice, with a body weight of 22 to 24 g were provided by the Breeding Laboratory of Experimental Animal Institute, the Chinese Academy of Medical Science (CAMS), license number is scxk-2004-0001.

Tumor cell line

Lewis lung cancer is a solid tumor. It was provided by the Institute of Materia Medica, Chinese Academy of Medical Science.

Inoculation of the tumor

The healthy tumor-bearing animals, following 10 days of inoculation with the tumor, were sacrificed by cervical dislocation. Their coats were thoroughly rinsed with 75% alcohol and the tumors removed using standard sterilization procedures. After washing off the blood, the tumors were placed in a stroke-physiological saline solution (SPSS), and a 1:3 cell suspension prepared using a tissue homogenizer. Each mouse was given a 0.2 ml hypodermic inoculation of the tumor suspension below the right axilla.

Grouping and administration

On the day following inoculation of the mice, they were divided into 5 groups, i.e. 3 GOG -treatment groups, receiving a high, medium or low GOG dose (10.0 mg/kg, 7.5 mg/kg, 5.0 mg/kg), a positive-control group (CDDP, 1.5 mg/kg) and a negative-control group. Intraperitoneal injections were conducted once a day, with a volume of 0.5 ml/per mouse for 8 days in all groups. Isovolumic SPSS was given to the negative-control group. The animals were dissected 24 h after the last injection.

Observation index

After the mice were sacrificed, the tumors were removed, cleaned, weighed and a tumor control rate cal-

culated. The tumor control rate (%) = $(1 - \text{mean tumor weight in a treated group}) / (\text{mean tumor weight in the negative control group}) \times 100\%$.

Anatomical dissection

The skin and thoracic bones were cut open along the median line of the neck and chest-wall and the lungs exposed to detect metastasis. Hypo-laryngeal excision was performed, the tissue including the trachea, bronchus and lungs were removed from the thoracic cavity, and were put on a filter paper-padded flat plate. After blotting off the blood, the tissues and animals were weighed.

Immunohistochemistry and H&E staining

The primary tumor and pulmonary metastatic tumors were embedded in paraffin blocks followed by immunohistochemical assays and / or H&E staining. The expression of the vascular endothelial growth factor (VEGF), apoptotic-related proteins Bcl-2, Bax and mutant p53 in the primary tumors of each group were determined and compared. The histomorphological characteristics of the metastasis were observed, and were respectively compared with those in the negative and the positive-control groups, in order to assess the anti-tumor and anti-metastatic effects of GOG.

Judgment of immunohistochemical results

VEGF localized in the cytoplasm, Bcl-2 and Bax appeared at the cell membrane and cytoplasm and p53 was found in the nucleus. If both the cytoplasm and the nucleus showed expression, the cells would be identified as having nuclear expression. Five high-power fields were randomly counted for each microscopic section ($\times 400$), with counting of 200 cancer cells in each area, and the percentage of the positive cells was calculated. Based on an analysis of both the percentage of positive cells and scoring of the coloration intensity, a total score was calculated. Scores of positive cells ranged from 0 to 4. A score of 0 meant an absence of any expression, a score of 1 meant the percentage was less or equivalent to 25%, 2 if between 26% to 50%, 3 if between 51% to 75% and 4 if more than 75%. The score for coloration intensity ranged from 0 to 3. Staining intensity was based on the color reaction of most cells, i.e. 0 was the score for achromatic color, 1 for yellowish, 2 for brownish yellow and 3 for brown. The final score was graded into 4 divisions, based on the total scoring after addition of the 2 indices, i.e. the score 0 to 1 for negative (-), 2 to 3 for weakly positive (+), 4 to 5 for moderately positive (++) and 6 to 7 for strongly positive (+++). The medium intensity and over could be identified as high expression.

Statistical analysis

The *t*-test, χ^2 test and Fisher precise probabilistic methods were used. SPSS10.0 statistical software analysis and treatment were conducted for all experimental data.

Results

Results of the tumor suppression study

The analysis showed that GOG had a significant anti-tumor effect within a definite dosage, and a preliminary anti-metastatic role (Table 1). In the high-dose group, the tumor-control rate was 49.6% ($P < 0.01$), and the lung weight and pulmonary coefficient were markedly lower in the high-dose group compared to those in the negative-control group ($P < 0.01$). On the 3rd day after administration, however, the mice were somewhat cachectic, with slight piloerection, and death occurred in two of the experimental animals. Subcutaneous edema and small scutiform hemorrhagic foci of the liver were found upon dissection, and a large amount of amber-color bile retention was seen in the small bowel.

In the medium-dose group, the tumor-control rate was 51.1% ($P < 0.01$). Although the pulmonary coefficient of this group was less than that of the negative-control group, there was no significant difference between the two groups ($P > 0.05$). One animal in this group died during treatment, however, no overt abnormality was found upon dissection.

The tumor-control rate of the low-dose group was 38.3% ($P < 0.01$), and the lung weight and pulmonary coefficient were obviously lower than those in the negative-control group ($P < 0.01$).

Toxicity of the GOG was confirmed during the tests. Animal deaths were found in both the high and the medium-dose groups, and a 7.4% and 5.4% decrease of the bodyweight of animals took place respectively. In the positive-control group with a dose of 1.5 mg/kg CDDP, 8 injections were given, and the tumor-control rate was 63.6%. A decrease in the body weight of the mice

reached 16.0% after medication, nevertheless no animal deaths occurred (Table 1.).

H&E staining results

H&E staining showed that the shape and size of the cells in the tumors of the lung tissue in each group were in accordance with the subcutaneous in situ tumor cells.

Immunohistochemical results

As shown in Table 2, there was a significant difference in comparing VEGF expression between all GOG dosage groups and the negative-control group ($P < 0.05$), i.e. the VEGF expression in all 3 groups treated with GOG was significantly inhibited (Figs.1 and 2). Bcl-2 expression was significantly lower in all GOG treated groups compared to the negative-control group, (5 and 7.5 mg/Kg, $P < 0.01$; 10 mg/Kg, $P < 0.05$) (Figs.3 and 4). The Bax expression was significantly higher in the large and medium-dose GOG groups compared to the negative-control group, and the Bax expression was also higher in the small-dose group, compared to the negative control group. However, no statistical difference was found in the comparisons ($P > 0.05$) (Figs.5 and 6).

The mutant p53 expression was significantly lower in the groups of various GOG doses compared to the negative control group, (10 mg/Kg, $P < 0.05$; 5 and 7.5 mg/Kg, $P < 0.01$) (Figs.7 and 8).

Discussion

Saponins are one of the important bioactive ingredients in Chinese herbal medicines. Many commonly-used Chinese crude drugs, such as ginseng, milkvetch root, Radix glycyrrhizae, Chinese thoroughwort, Dwarf lily-

Table 1. The experimental results of the tumor suppression and anti-metastasis of GOG on Lewis lung cancer.

Group	Dose (mg/kg)	Number of animals		Tumor weight (g) $\bar{x} \pm s$	Tumor control rate (%)	P	Lung coefficient* $\bar{x} \pm s$	P
		Start	End					
Negative control	-	20	20	2.12 ± 0.56	-	-	0.085 ± 0.013	-
CDDP	1.5	11	11	0.77 ± 0.38	63.6	< 0.01	0.073 ± 0.012	< 0.05
GOG	10.0	14	12	1.07 ± 0.35	49.6	< 0.01	0.072 ± 0.006	< 0.01
	7.5	13	12	1.04 ± 0.46	51.1	< 0.01	0.077 ± 0.014	> 0.05
	5.0	13	13	1.31 ± 0.38	38.3	< 0.01	0.069 ± 0.009	< 0.01

*Grams of the lung tissue per 10 g of the murine body weight.

Table 2. Statistical analysis of the VEGF, Bcl-2, Bax and p53 expressions in all groups (positive rate %).

Group	Dose (mg/kg)	VEGF	Bcl-2	Bax	p53
Negative control	-	75.0	80.0	25.0	85.0
CDDP	1.5	72.7	63.6	45.5	54.5
GOG	10.0	33.3*	33.3*	75.0*	41.7*
	7.5	33.3*	25.0**	66.7*	33.3**
	5.0	30.8*	30.8**	61.5	30.8**

*, Compared with negative control group, $P < 0.05$; **, Compared with negative control group, $P < 0.01$

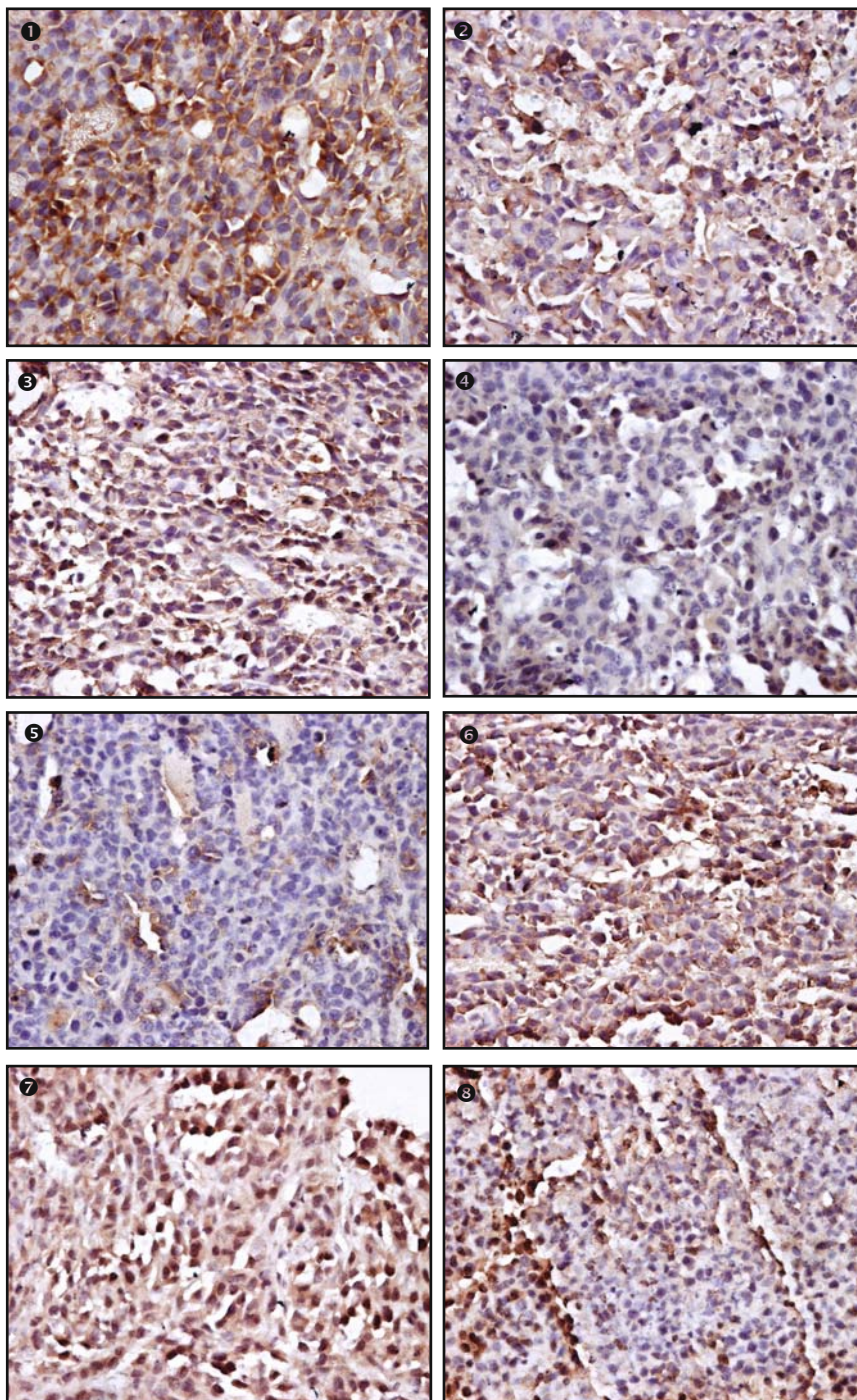


Fig.1. Expression of VEGF in tumors of the negative control group.
 Fig.2. Low expression of VEGF in tumors of the large-dose GOG group.
 Fig.3. Expression of Bcl-2 in tumors of the negative control group.
 Fig.4. Low expression of Bcl-2 in tumors of the large-dose GOG group.
 Fig.5. Expression of Bax in tumors of the negative control group.
 Fig.6. High expression of Bax in tumors of the large-dose GOG group.
 Fig.7. Expression of P53 in tumors of the negative control group.
 Fig.8. Low expression of P53 in tumors of the large-dose GOG group.
 Immunohistochemical S-P methods × 400 for Figs.1~8.

turf tuber and Common anemarrhena rhizome etc., contain a large amount of saponin. Saponins are composed of two parts, i.e. the sapogenin and the saccharide chain. The structure of the aglycone can be divided into steroidal saponins and triterpenoid saponins (TS). The TS is composed of a triterpenoid sapogenin and carbohydrate, aldonic acid, and other organic-acid compositions. The development of saponin chemistry has fostered the understanding of its bioactivity, especially anti-tumor effects, hypoglycemic activity, immunological regulation, and prevention and treatment of cardiovascular diseases etc. These activities have increasingly aroused interest all over the world, and use of triterpenoid saponin has

become one of the most active and quickly developing fields in natural product research.

TS can improve nonspecific immunity, enhance secretion of some cytokines, and activate immunocytes, thus strengthening prevention and treatment of diseases, anti-aging and anti-cancer activity in humans^[3]. Panaxoside, for instance, has shown to increase the level of serum complement and specific antibody^[4], and the TS in the *Largetrifolioliosus Bugbane Rhizome* has several physiological functions, such as an anti-tumor effect, inhibition of nucleoside transport, and endocrine regulation etc.^[5] GOG belongs to the saponin class of compounds, which is extracted from the *Gypsophila oldhamiana* Miq (i.e.

tumble-weed), a Caryophyllaceae plant used in Chinese herbal medicines. GOG may be developed into a new anticancer drug, but there is a lack of available studies and data. So in addition to our study on the anti-cancer effects of GOG, preliminary multi-target and multifactorial observations of the anti-cancer effects are also needed. GOG had a definite anti-tumor effect on Lewis lung cancer with an effective dose in the range of 5.0 to 10.0 mg/kg.

VEGF is a vascular endothelial cell (VEC) mitogen with a high degree of specificity, and as far as it is known, the most powerful and direct effect on VEC of any growth factor. It enhances tumor metastasis by stimulating angiopoiesis. Since the vessel walls are thin and the tumor destroys the basal membrane, there are fissures between the endothelial cells. In addition, VEGF induces the VEC to produce enzymes that degrade the matrix, permitting exfoliation of the tumor cells, and invasion of the blood vessels resulting in a distant metastasis^[6,7]. In our study, we investigated the possible anti-metastatic mechanism of GOG. VEGF expression was found to be significantly lower in the GOG-treated groups compared to the negative-control group ($P < 0.05$), indicating that GOG has a definite anti-angiogenic effect on Lewis lung cancer by causing inhibition of tumor growth and metastasis.

Alteration of apoptosis can remarkably extend the survival time of cells, influence the accumulation of gene mutations and reduce cellular immunological surveillance^[8]. The Bcl-2 gene is one of a family of apoptotic regulatory genes, which has a significant impact on the onset, progress and tumor therapy^[9]. Bax and Bcl-2 are a pair of positive and negative apoptotic regulatory genes. Bax may form a heterodimer with the Bcl-2 protein, resulting in Bcl-2 inactivation, thus promoting apoptosis. Bcl-2, a suppressor gene of apoptosis, showed significantly lower expression in the GOG treated group compared to the negative-control group, and expression of Bax, a promoter of apoptosis was significantly higher in groups receiving the high and medium-dose of GOG. In the negative-control group, there was a high expression of the Bcl-2 protein and a low expression of Bax. Furthermore, the gross tumor volume was reduced in the treated groups. The results suggest that GOG treatment can alter the expression of both Bcl-2 and Bax proteins, reduce the expression of the Bcl-2 protein and/or raise the Bax expression, promoting tumor apoptosis. Therefore therapy with GOG is of significant importance to the treatment of Lewis lung cancer.

P53 gene mutations closely relate to a variety of tumorigenesis. Results of our study indicated that mutant P53 and VEGF expressions were significantly higher in the negative -control group than in the GOG-treated groups, suggesting that there is a definite relationship between mutant P53 and VEGF expressions. It was reported that^[10,11] the wild-type P53 gene can inhibit the V-Src-induced up-regulated VEGF expression, and that mutant P53 genes may increase the VEGF expression

to promote angiogenesis. Moreover, since the wild-type P53 is located upstream of Bax and Bcl-2 in the apoptotic pathway, Bcl-2-induced apoptosis can be down-regulated by up-regulating Bax, and mutant P53 may delay apoptosis. In the GOG-medicated groups, by down-regulating mutant P53, VEGF and Bcl-2 expressions, Bax expression would be up-regulated, apoptosis of the tumor cells promoted, and angiogenesis of the tumor inhibited. This chain of events would depress the progress of Lewis lung cancer, and inhibit metastasis formation. Although the pulmonary coefficient was less in the medium-treated group compared to the negative-control group, there was no significant difference between the two groups ($P > 0.05$). These results remain for further investigation.

In this study, a general toxic reaction in the mice might relate to the saponin character of GOG, because the high level of medication resulted in an in vivo hemolysis. In conclusion, GOG administration can depress the growth and metastasis of Lewis lung cancer by inhibiting tumor angiogenesis and inducing multi-target effects such as apoptosis etc.

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