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Anti-tumor effect and influence of *Gekko gecko Linnaeus* on the immune system of sarcoma 180-bearing mice

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Received August 8, 2008; Accepted March 16, 2009

DOI: 10.3892/mmr_00000139

Abstract. *Gekko gecko Linnaeus* (GgL) is an extract used in traditional Chinese medicine. In the present study, we examined the anti-tumor activity of GgL and its effect on the immune system of mice. Sarcoma 180-bearing mice were used as the animal model, and cisplatin was applied as the positive control drug. The mice were randomly divided into six groups, and each group was treated with a different drug or drug concentration. The effects of GgL were evaluated based on its anti-tumor activity and prolongation of the lifespan, the lymphocyte transformation rate and pathological changes observed in the tumors. The results suggest that GgL has anti-tumor activities and up-regulates the immune system in a dose-dependent manner. This study provides original data related to the anti-tumor and immune up-regulating function of GgL.

Introduction

Chemotherapy is one of the main clinically-applied cancer treatments. Most chemotherapeutic drugs are cytotoxic; they not only kill tumor cells, but also simultaneously normal cells, causing numerous side effects (1,2). With the ongoing exploration of traditional medical resources, doctors are placing increasing emphasis on traditional medicine-assisted treatment, which has shown an applicable foreground (3,4).

Gekko gecko Linnaeus (GgL) is an extract commonly used in traditional Chinese medicine that is considered to be effective based on clinical and medical scientific research. In China, clinical and basic pharmacological research on GgL has been extremely active since the beginning of this century. GgL is used to treat liver and kidney deficiencies, and to relieve cough and asthma (5). It also has anti-tumor activities, on which little research has been conducted to date.

We set up an ovarian cancer model using sarcoma 180-bearing (S_{180}) mice, with which we investigated the effects of various doses of GgL on tumor formation and the immune system. The results provide a foundation for further toxicological and clinical research on GgL.

Materials and methods

Tumor cells and implantation procedure. S_{180} tumor cells were provided by the Tumor Specialty Hospital of Harbin Medical University. The cells were injected into the hind leg muscle of 108 4-week-old female nude BALB/c mice (2-3x10^5 cells per animal). The study protocol was approved by the Ethics Committee of Harbin Medical University, P.R. China.

Group formation and drug administration protocols. Tumor-implanted mice were randomly divided into six groups, treated as follows: group 1, sodium chloride solution; group 2, low-dose GgL (2.4 g/kg); group 3, high-dose GgL (12.4 g/kg); group 4, cisplatin + sodium chloride solution; group 5, cisplatin + low-dose GgL (2.4 g/kg); group 6, cisplatin + high-dose GgL (12.4 g/kg). GgL was administered i.g. at 0.4 mg/day for 10 days. Cisplatin was administered i.p. at 7 mg/kg just once, 24 h after tumor cell injection.

Sample collection. On day 11, the surviving mice were sacrificed. The tumor mass was immediately removed and weighed, and the anti-tumor effect was evaluated. Spleen samples were obtained and the spleen index was calculated, and the lymphocyte transformation rate was measured using the MTT assay (6). Optical and electron microscopy were used to determine the pathological characteristics and changes in the tumor and spleen samples.

Analysis of the anti-tumor rate. Tumors were weighed and the t-test was performed using the average weight of each group. The anti-tumor rate was analyzed using the formula: anti-tumor rate = $(W_c-W_d)/W_d$ x 100%, where W_c is the weight of the tumor mass of the control group and W_d is the weight of the tumor mass of the experimental drug groups.

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Key words: Gekko gecko Linnaeus, traditional medicine, anti-tumor, immune

Table I. Prolongation of lifespan by *Gekko gecko Linnaeus*.

Group	No. of animals	Survival time (days)	Life extension rate (%)
Sodium chloride solution	18	13.22±4.99	-
Low-dose GgL	18	16.78±4.98	21.22
High-dose GgL	18	18.06±4.09	26.80
Cisplatin + sodium chloride	18	19.17±3.27	31.04
Cisplatin + low-dose GgL	18	20.83±4.41	36.53[a]
Cisplatin + high-dose GgL	18	21.89±4.20	39.61[a]

[a]p<0.05 compared with the sodium chloride group. Survival time is presented as the means±SD.

Table II. Anti-tumor effect of *Gekko gecko Linnaeus*.

Group	No. of animals	Weight (g)		Tumor weight (g)	Tumor inhibitory rate (%)
		Start	End		
Sodium chloride solution	8	18.025±0.419	21.825±1.076	5.013±0.463	-
Low-dose GgL	8	18.063±0.941	23.825±2.981	3.713±0.203	25.94
High-dose GgL	7	18.043±1.859	25.300±1.269	3.186±0.195	36.44
Cisplatin + sodium chloride	7	18.043±0.408	14.329±0.507	2.057±0.190	58.96
Cisplatin + low-dose GgL	8	18.125±1.135	19.888±2.220	1.863±0.167	62.84[a]
Cisplatin + high-dose GgL	7	18.129±0.346	20.329±0.331	1.729±0.160	65.51[a]

[a]p<0.01 compared with the sodium chloride group. Weights are presented as the means±SD.

Analysis of the life extension rate. As a control, a second set of 108 4-week-old female nude BALB/c mice, injected with control cells that had been cultured normally, was randomly divided into 6 groups and subjected to a drug administration regime identical to the one described above. The quality of life and lifespan of the mice were noted and compared to those of the experimental groups. The life extension rate was calculated using the formula: life extension rate = $(T_d - T_c)/T_d$ x 100%, where T_d is the lifespan of the experimental groups and T_c is the lifespan of the control group.

Spleen weight and calculation of the spleen index. Spleen samples were obtained aseptically and weighed. The spleen index was calculated using the formula: spleen index = spleen weight/body weight.

Calculation of the lymphocyte transformation rate. MTT (Invitrogen) was used to calculate the lymphocyte transformation rate according to the manufacturer's instructions. On day 11, the surviving mice were sacrificed and sterilized with 75% alcohol. Spleen samples were collected and placed in petri dishes with a glass cover slip containing Hank's solution (Invitrogen). The samples were then smoothly ground with a stainless steel sieve to produce a single-cell suspension fluid, which was transferred to a closed aseptic test tube. Cells were washed in Hank's solution then centrifuged at 1100 rpm for 10 min. This step was repeated three times, then the cells were resuspended in Hank's solution. The number of cells was adjusted to $1x10^6$/ml. The cell suspension fluid was distributed in 96-well petri dishes (100 μl/well in 4 wells). Concanavalin A (ConA) (5 μg/ml) (Invitrogen) and lipopolysaccharide (LPS) (10 μg/ml) (Invitrogen) were respectively added to 2 wells of the dish. The other 2 wells served as the control. The cells were incubated at 37˚C with 5% humidified CO_2 for 36 h. Four hours before the end of the incubation period, 50 μl MTT (5 g/l) was added to each well. Following incubation, the medium was replaced with acid isopropanol and the mixture was agitated until the purple crystals dissolved. Absorbance (OD) was measured at 570 nm using a 550 Microplate reader, and the lymphocyte transformation rate was calculated.

Statistical analysis. Data were analyzed using SPSS statistical analysis software.

Results

Prolongation of the lifespan. Following drug administration, the respiration and activity of the mice in the tumor groups were normal, with the exception of group 1, which was administered a sodium chloride solution. Mice in this group were weak and short of breath; they were reluctant to move and their fur was thin and dark. The average survival time of groups 5 and 6 (cisplatin + high- and low-dose GgL) was markedly increased compared to that of group 1 ($p<0.05$), but was no different compared to the survival time of group 4 (cisplatin + sodium chloride solution) ($p>0.05$) (Table I).

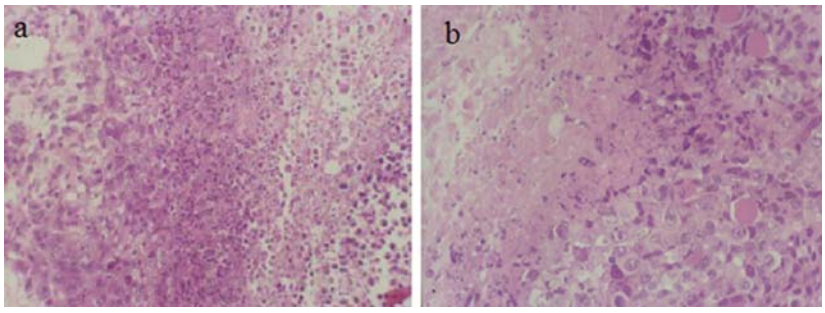

Figure 1. Representative lymphoid tumor of the (a) cisplatin + sodium chloride group (x200) and (b) cisplatin + GgL group (x400) using optical microscopy.

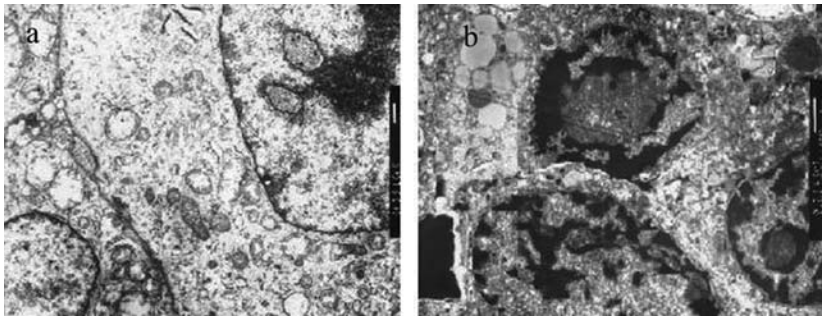

Figure 2. Representative lymphoid tumor of the (a) cisplatin + sodium chloride group (x10K) and (b) cisplatin + GgL group (x8000) using electron microscopy.

Table III. Effects on the immune system of *Gekko gecko Linnaeus*.

Group	No. of animals	Average spleen weight (g)	Spleen index
Sodium chloride solution	8	1.953±0.017	8.98±0.78
Low-dose GgL	8	1.979±0.019	8.31±0.10
High-dose GgL	7	2.030±0.048	8.01±0.25[a]
Cisplatin + sodium chloride	7	1.840±0.027	12.87±0.32
Cisplatin + low-dose GgL	8	1.911±0.036	9.61±0.15
Cisplatin + high-dose GgL	7	1.913±0.011	9.41±0.37

[a]$p<0.05$ compared with the cisplatin + sodium chloride group. Data are presented as the means±SD.

Anti-tumor activities. Following treatment with cisplatin and GgL, the tumor growth rate was markedly reduced. The anti-tumor rates of group 3 (high-dose GgL) and group 4 (cisplatin + sodium chloride solution) were 36.44 and 58.96%, respectively, and were significantly different compared to group 1. The anti-tumor rates of groups 5 and 6 were 62.84 and 65.51%, respectively, and were significantly different compared to group 1. However, the anti-tumor rates of groups 5 and 6 were not significally different compared to group 4. The results indicate that GgL has an anti-tumor effect which tends to be enhanced with an increase in its concentration, and that it can strengthen the curative effect of cisplatin (Table II).

Lymphoid tumor pathology. Optical and electron microscopy were used to examine the characteristics of the tumor cells. Using optical microscopy, groups 5 and 6 were observed to have a few tumor cells mixed with necrotic foci in the tumor mass, along with large areas of solidified necrosis (Fig. 1). Using electron microscopy, apoptosis was observed in the tumor cells of groups 5 and 6. In addition, the nuclei appeared solidified and shrunken, and the nuclear membranes were incomplete. Mitochondrial vacuolation was observed, and the endoplasmic reticulum was clearly visible (Fig. 2). These results indicate that tumor necrosis was more severe in groups 5 and 6 than in group 4.

Spleen weights and index. The spleens of mice in groups 2 and 3 (low- and high-dose GgL) were heavier than those of mice in group 1, while the spleens of mice in group 1 were heavier than those of group 4. The spleens of mice in groups 5 and 6 were also heavier than those of group 4, although without statistical significance. Additionally, the spleen index of group 3 was significantly lower than that of group 4, and was even slightly lower than that of group 1. This indicates that GgL strengthens the immune system, an effect which is enhanced with an increase in its concentration (Table III).

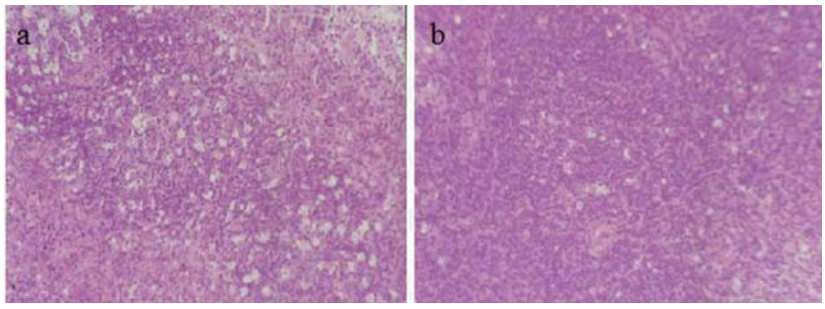

Figure 3. Representative spleen of the (a) cisplatin + sodium chloride group (x200) and (b) cisplatin + GgL group (x200) using optical microscopy.

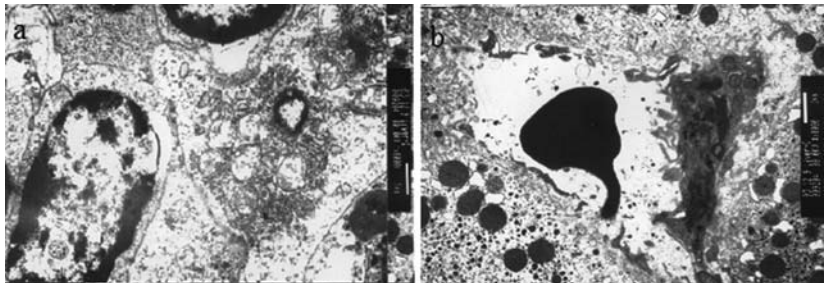

Figure 4. Representative spleen of the (a) cisplatin + sodium chloride group (x10K) and (b) cisplatin + GgL group (x8000) using electron microscopy.

Table IV. Lymphocyte transformation.

Group	No. of animals	ConA	LPS
Sodium chloride solution	8	1.176±0.014	1.150±0.019
Low-dose GgL	8	1.200±0.013[a]	1.170±0.011[a]
High-dose GgL	7	1.220±0.022[b]	1.200±0.014[b]
Cisplatin + sodium chloride	7	0.967±0.060	0.760±0.024
Cisplatin + low-dose GgL	8	1.050±0.018	1.070±0.017
Cisplatin + high-dose GgL	7	1.130±0.026	1.150±0.018

[a]$p<0.05$ and [b]$p<0.01$ compared with the cisplatin + sodium chloride group. Data are presented as the means±SD. ConA, Concanavalin A; LPS, lipopolysaccharide.

Spleen pathology. Optical and electron microscopy were used to examine changes in the spleen samples. Using optical microscopy, lymphocyte apoptosis and activated macrophages were observed in groups 5 and 6, though to a lesser degree than in group 4 (Fig. 3). Using electron microscopy, groups 5 and 6 were observed to have well developed spleen cells, and red pulp and splenic corpuscles with normal structures. Nuclei were normal, and nucleons were clear. A large number of well-developed mitochondria were observed around the nucleus, and only a few catkin-like foci were present in the cytoplasm. In contrast, in group 4, the nuclear envelope was unclear, heterochromatin was marginalized to the edge of the nucleus and nuclei were solidified and shrunken (Fig. 4).

Lymphocyte transformation. With the addition of induction reagents, lymphocytes were converted to lymphoblasts, which proliferated and released lymphoid factors. As induction reagents, ConA for the most part induced the proliferation of T lymphocytes, while LPS mainly induced the proliferation of B lymphocytes. Cisplatin had a strong inhibitory effect on the proliferation of T and B lymphocytes in the spleen tissues ($p<0.01$), while GgL promoted their proliferation. There was significant difference in lymphocyte proliferation between group 1 and groups 2 and 3, with higher concentrations of GgL having an enhanced effect. These results indicate that GgL can markedly strengthen the immune response induced by T or B lymphocytes (Table IV).

Discussion

Malignant ovarian tumors are the third most commonly occuring female reproductive system tumors, but are first in terms of mortality (7). This is primarily due to the lack of a specific tumor marker for the cancer, and to the fact that patients in the early stages of the disease don't present obvious symptoms, making early diagnosis a challenge (8).

At the time of diagnosis, approximately 70% of patients are in the late stages of ovarian cancer. However, with the rapid

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nent of new medical techniques, including the e of B ultrasounds, CT scans, peritoneoscopy, MRI and PET scans, and laboratory examinations being conducted in all fields, the number of cases of ovarian cancer diagnosed and treated at an early stage has the potential to increase, with associated improvements in survival rate (9,10).

In China, malignant ovarian tumors are most commonly treated by surgery, with the additional administration of chemotherapy and radiotherapy regimes. However, the development of biological therapy (immunotherapy) would greatly benefit patients (11). Tumor occurrence and development are associated with the down-regulation of the immune system (12). In normal situations, cells and the body defense system are balanced. When this balance is disrupted, tumors form, proliferate and metastasize. Thus, theoretically, if the unbalanced immune system is returned to its normal state, tumor cell proliferation will be inhibited.

The immune system of ovarian cancer patients is unbalanced; during the later stages of the disease, almost all normal immune reactions are suppressed, so the drugs used in chemotherapy not only kill tumor cells, but also destroy the body's immune system (13). As the extent of immune system down-regulation varies from patient to patient, monitoring immunochemical data throughout treatment is necessary to guide and restructure the therapeutic plan and improve patient prognosis.

Chemotherapy is one of the most commonly applied and important cancer treatments. However, it is rejected by many patients due to its serious side effects, negatively affecting treatment. The administration of traditional Chinese medicines in conjunction with chemotherapy has the potential to relieve side effects and toxicity, and to enhance the function of the immune system (14,15).

In the present study, GgL was applied as a biological mediator of immune response. We studied the anti-tumor effect of different doses of GgL on tumors and the spleen, an organ indicative of the state of the immune system. GgL promoted the apoptosis of tumor cells, improved the quality of life and prolonged the lifespan of the mice in our model, suggesting it inhibits tumor proliferation and improves the balance of the immune system.

The functioning of the immune system is closely related with the condition of organs such as the spleen. Some immunosuppressants, such as cisplatin, can cause spleen atrophy, while immune enhancers can increase spleen weight. Thus, the spleen can serve as an experimental indicator of the condition of the immune system.

The results of the present study indicate that GgL significantly reduced a spleen index abnormally increased by the administration of cisplatin, even to the levels of a tumor-bearing group administered a sodium chloride solution. Additionally, GgL enhanced the transformation of T and B lymphocytes, suggesting that it inhibits tumor cell activity by strengthening the immune system. GgL may primarily affect the monitoring function of the immune system as well as T and B lymphocyte-mediated immune response. It is well known that activated T and B lymphocytes are capable of directly killing tumor cells. In addition, they can produce a series of lymphoid factors that, together with macrophages, induce apoptosis and inhibit the proliferation of tumor cells. This is one aspect of cancer immunotherapy.

In conclusion, GgL can be used as a supplementary drug in clinical chemotherapy for malignant tumors, functioning as a tumor inhibitor and up-regulating the immune system. In further experiments, we will continue to investigate the functions and mechanism of GgL, with the aim of demonstrating its utility as an effective cancer treatment.

Acknowledgements

This work was supported by the First Affiliated Hospital of Harbin Medical University Scientific Research Fund (no. 2007092), the Scientific Fund of the Department of Health of Heilongjiang Province (no. 2007-205) and by the Heilongjiang Natural Science Foundation (no. D200805).

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