Effects of Feijining Decoction on vascular endothelial growth factor protein expression and changes of T cell subsets in Lewis lung carcinoma-bearing mice

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Abstract. Angiogenesis is crucial for cancer growth and metastasis. T cells are also key members of the adaptive immunity against tumorigenesis. The aim of the present study was to observe the effects of Feijining Decoction (FJND) on vascular endothelial growth factor (VEGF) protein expression and T cell subsets [cluster of differentiation 4^+($CD4^+$) and $CD8^+$ T lymphocyte] in Lewis lung carcinoma (LLC)-bearing mice. C57BL/6J mice were subcutaneously implanted with LLC cells. Forty carcinoma-bearing mice were randomly assigned to four groups (10 animals/group). The control group (CG) were the untreated group, the cisplatinum (DDP) group (DG) mice were treated with DDP, the FJND group (FG) were treated with FJND and the FJND + DDP group (FDG) were treated with FJND and DDP. Western blot and flow cytometry were used to evaluate the VEGF protein expression of tumor tissue and T cell subsets of the spleen. Spontaneous activity in 5 min was observed by the photoelectric counting method. DDP + FJND (FDG group) markedly inhibited tumor growth compared to the DG mice. The protein expression of VEGF was significantly downregulated in the carcinoma of FG mice compared to CG mice. VEGF protein expression was significantly reduced in FDG compared to DG mice. In the FG mice, the splenic $CD4^+$ and $CD4^+/CD8^+$ cells were significantly increased compared to the CG mice, and the splenic $CD4^+$ cells in the FDG mice were significantly increased compared to the DG group. In conclusion, FJND can inhibit tumor growth by downregulating VEGF protein expression and improving the immune function.

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Introduction

Angiogenesis, the growth of new blood vessels, plays an important role in tumor development and metastasis. Antiangiogenic therapies have been demonstrated to inhibit tumor growth and prolong progression-free survival and/or overall survival. Vascular endothelial growth factor (VEGF) is thought to be the major regulator of physiological and pathological angiogenesis. A decrease of the serum VEGF level will directly influence the downstream angiogenic process (1-3).

Chai Hu Long Gu Mu Li soup, a classic formula of Traditional Chinese medicine (TCM), was described in a TCM monograph (Shang Han Lun) by Ji Zhang, a Han dynasty physician (A.D. 150-219). Feijining Decoc-tion (FJND) was developed by Professor Dongfeng Yin [Affiliated Hos-pital of Liaoning University of Traditional Chinese Medicine (AHTCM), Liaoning, China] from Chai Hu Long Gu Mu Li soup, and has been used to treat lung cancer for decades. Our previous experimental study confir-med that FJND inhibited the growth of A549 cells, which is possibly associated with decreased VEGF expression (4).

According to recent research, VEGF also exerts a systemic influence on immune cell development and function. In cancer, VEGF is present at high levels in the tumor and the systemic circulation. Elevated levels of circulating VEGF inhibit T-cell immune responses (5,6). Immunotherapy is a central component of numerous cancer treatment regimens as well.

Ginseng, a prime ingredient in FJND, is also particularly popular as a treatment among cancer patients, since multiple studies have associated the consumption of ginseng with cancer prevention and treatment, and with an improved well-being (such as cancer-related fatigue and immunity) during cancer therapy (7,8). Ginsenoside Rg3, an extract from ginseng, has demonstrated anti-cancer activity *in vitro* and *in vivo* with relatively low toxicity, particularly on vessels or angiogenesis in tumors, and it selectively suppressed VEGF expression (9,10). In order to explore the an-titumor effects

of FJND, the present study was performed on Lewis lung carcinoma (LLC)-bearing mice.

Materials and methods

Animals and cells. Sixty male C57BL/6 mice weighing 20±2 g were used in the study. The mice were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China; Certificate of Conformity: SCXK Jing 2004-0001). The animals were maintained in a pathogen-free facility (22±2˚C, 55±5% humidity) and a 12-h light/dark cycle with lights on from 07:00 to 19:00 h daily. Food and water were provided *ad libitum*. All the procedures on treating mice were performed according to the Animal Care Guidelines issued by the Ministry of Science and Technology of China and the Animal Care Committee of Liaoning University of Traditional Chinese Medicine approved the protocols.

LLC cells were obtained from the Department of Immunology, College of Basic Medical Science, China Medical University (Wuhan, China) and maintained in Dulbecco's modified Eagle's medium supplemented with 100 ml/l fetal bovine serum, penicillin ($1x10^5$ U/l) and streptomycin (100 mg/l) with 5% CO_2 at 37˚C in a humidified environment.

Chemicals and reagents. Cisplatinum (DDP; lot no. 6120251DB) was obtained from Qilu Pharmaceutical Co., Ltd., Jinan, Shandong, China. FJND consisted of 13 herbal materials, including *Radix Bupleuri* (10 g), *Scutellaria baicalensis Georgi* (15 g), *Rhizoma Pinelliae* (10 g), *Ginseng* (10 g), *Fossilia Ossis Mastodi* (25 g), *Concha Ostreae* (25 g), *Psuedobulbus Cremastrae* (15 g), *Zedoary Rhizoma* (15 g), *Fritillariae Thunbergii* (15 g), *Radix Platycodi* (15 g), *Hedyotis Diffusa* Willd (20 g), Indian buead (15 g) and *Radix Glycyrrhizae* (10 g). All were purchased from the Hospital Pharmacy of AHTCM. FJND was extracted by a routine method which is used in the Key Laboratory of Department of Integrated Traditional Chinese and Western Medicine, Peking University School of Oncology, as reported previously (11-13).

LLC-bearing mice. Solid-type LLC was prepared by subcutaneous transplantation of $1x10^7$cells (0.2 ml) into the armpits of 60 C57BL/6 mice. The tumor volume was determined every two days by direct measurement with calipers and calculated using the formula: [Width^2 (mm^2) x length (mm)]/2. The treatment was initiated while all the tumor volumes were >100 mm^3 in ≥40 mice. Forty mice were randomly assigned to four groups (10 animals/group); control (CG), DDP (DG), FJND (FG) and FJND + DDP groups (FDG). A 0.9% NaCl solution (0.4 ml/20 g) was administered intragastrically once daily for two weeks in CG and DG mice, and a 0.9% NaCl solution (0.1 ml/20 g) was administered intraperitoneally once daily for 1, 3 and 5 days in CG and FG mice. DG and FDG mice were provided DDP (0.1 ml, 0.1 mg/20 g) intraperitoneally once daily for 1, 3 and 5 days. FG and FDG mice were administered FJND (0.4 ml, 0.62 g/20 g) intragastrically on the same schedule. The mice were treated daily for two weeks and sacrificed on day 15.

Spontaneous activity, tumor weight, thoracic gland and spleen index. Spontaneous activity in 5 min was observed by the photoelectric counting method and all the mice were weighed every other day. Tumor, thoracic gland and spleen tissues were cut to calculate the inhibitory rate (IR), thoracic gland index and spleen index. The IR was calculated as: [(Average tumor weight in the CG group - average tumor weight in the treatment group) / average tumor weight in the control group] x 100%. The thoracic (spleen) index was calculated as: (Thoracic (spleen) weight / body weight) x 100%.

Flow cytometry for $CD4^+$ and $CD8^+$. On day 15, the mice were sacrificed by cervical dislocation, and the spleen and thymus were quickly removed and weighed. The spleen tissues were gently teased to release cells by means of dissecting forceps in phosphate-buffered saline (PBS; pH 7.4). The lymphocytes were isolated using density gradient centrifugation (lymphocyte separation medium). The number of lymphocytes was measured using a Coulter counter. The cell concentration was adjusted to $1x10^{10}$ cells/l and 10 μl fluorescein isothiocyanate-labeled anti-mouse CD4 and CD8 (cat. .no. 11-0041-82 and cat.no. 12-0081-82, respectively; both from eBioscience, San Diego, CA, USA) was added to 100 μl of the cell suspension. After incubation for 30 min at 4˚C, the lymphocytes were rinsed three times with 1 ml PBS and centrifuged at 500 x g for 3 min. Subsequently, $CD4^+$ and $CD8^+$ were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Pathological section. A portion of tumor tissue was fixed by immersion in 10% buffered formalin (pH 7.4) for 24 h. The fixed tissue was dehydrated in graded ethanol, paraffin-embedded and sectioned at a thickness of 4 μm. The tumor histology of each group was observed under a light microscope.

Western blot analysis. VEGF protein expression was determined for each group by western blot analysis of the protein extracts obtained from the tumor tissue. The protein concentrations were determined by a bicinchoninic acid protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Proteins (30 μg) were separated by 12% SDS-PAGE, transferred to polyvinylidene fluoride and blocked with skimmed milk for 2 h at room temperature and incubated with the primary antibody (1:1,000; rabbit polyclonal antibody; BA0407; Wuhan Boster Biological Technology, Ltd., Wuhan, China) at 4˚C overnight. The next day the membranes were incubated with the secondary antibody for 1 h (1:5,000; peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L); cat. no. ZB-2301; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The targeted proteins were visualized by autoradiography. The grayscale was measured using the software BandScan 4.0 (Glyko, Inc., Novato, CA, USA). The relative densities of VEGF protein verses β-actin were calculated.

Statistical analysis. The values are presented as the mean ± standard deviation relative to the control values. Statistically significant differences from the control group were identified by one-way analysis of variance for the data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in body weight. The body weights of the DG mice were significantly reduced to 14.5±1.01 from 20±0.54 g compared to

Table I. Effects of Feijining Decoction (FJDN) on the body weight and spontaneous activity in Lewis lung carcinoma-bearing mice.

Groups	n	Body weights, g		Rearing and ambulating, counts/min
		Pre	Post	
CG	10	20±0.56	19.6±0.94	19±2.52
FG	10	20±0.47	22.5±0.83	25±2.13[a]
DG	9	20±0.54	14.5±1.01	2±1.98
FDG	8	20±0.87	16.8±1.24	12±0.87[b]

[a]P=0.031 compared to CG; [b]P=0.021 compared to DG. CG, control group; FG, FJDN group; DG, cisplatinum group; FDG, FJDN + cisplatinum group.

Table II. Effects of Feijining Decoction (FJDN) on tumor, spleen and thoracic index in Lewis lung carcinoma-bearing mice.

Groups	n	Tumor inhibitory rate	Spleen index,%	Thoracic gland index, %
CG	10	-	11.24	2.31
FG	10	26.1[a]	18.21[a]	2.79
DG	9	51.2	6.23	1.69
FDG	8	62.7[b]	14.34[b]	1.90

[a]P=0.027 compared to CG; [b]P=0.017 compared to DG. CG, control group; FG, FJDN group; DG, cisplatinum group; FDG, FJDN + cisplatinum group.

Table III. T cell subsets of the groups were measured and compared following therapy.

Groups	n	$CD4^+$, %	$CD8^+$, %	$CD4^+/CD8^+$
CG	10	12.04±3.01	16.57±3.49	0.73±0.34
FG	10	25.21±4.67[a]	17.59±2.15	1.43±0.54[a]
DG	9	9.01±1.78	16.58±3.72	0.54±0.31
FDG	8	15.37±2.34[b]	15.89±2.97	0.97±0.47[b]

[a]P=0.021 compared to CG; [b]P=0.013 compared to DG. CG, control group; FG, Feijining Decoction group; DG, cisplatinum group; FDG, Feijining Decoction + cisplatinum group.

16.8±1.24 from 20±0.87 g for the FDG mice. The DG mice became significantly less active in their cages, assessed by rearing and ambulating, compared to the active FDG mice, and the FG mice were more active than the CG mice (Table I).

Changes in tumor, spleen and thoracic index. Tumor growth and final tumor weight were significantly inhibited in the FDG mice. FJND significantly reduced the tumor compared to the CG mice. FJND may have enhanced the antitumor effects of

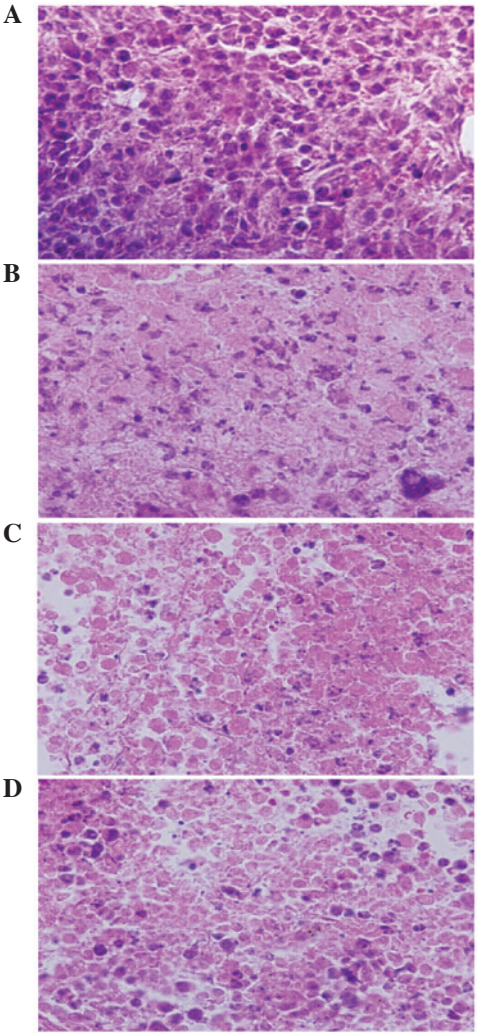

Figure 1. Morphological changes of tumor tissues by hematoxylin-eosin stain (magnification, x100) of the groups that were treated with different therapy. Nuclear staining of (A) CG mice compared to (B) FG mice. (C) Area of necrosis. (D) The number of plasmocytes and lymphocytes. CG, control group; FG, Feijining Decoction group.

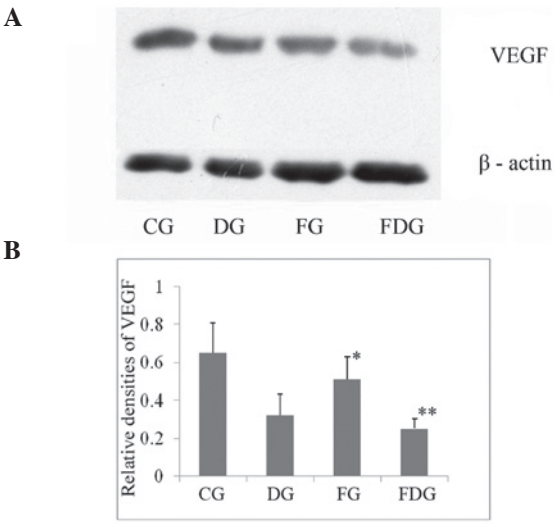

Figure 2. Image of VEGF protein expressions of the groups that were treated with different therapy. Data are shown as mean ± standard deviation. *P=0.018 compared to CG; **P=0.019 compared to DG. VEGF, vascular endothelial growth factor; CG, control group; DG, cisplatinum group; FG, Feijining Decoction group; FDG, Feijining Decoction+cisplatinum group.

cisplatin. FJND had a synergistic effect with cisplatin in the treatment of LLC-bearing mice (Table II). The spleen weights of FG mice were greater than those of the DG mice. The number of lymphocytes in the spleens of FG mice significantly increased compared to the CG mice. In DG, the number of lymphocytes in the spleens was significantly reduced compared to the FDG mice. However, FJND had no effect on thymus weight.

Morphological changes of tumor tissues. Following hematoxylin-eosin staining, the change of tumor structure was observed under a light microscope (Fig. 1). Tumor cells grew more productively, proliferated markedly atypical and had a deeper nuclear stain in the CG mice, as compared to the FG mice. In the tumor tissue of the FDG mice, there was a large necrosis area, and a scattered and abnormal small necrosis area. The plasmocytes and lymphocytes infiltrated with scattering or gathering around the necrosis area. The number of plasmocytes and lymphocytes in the DG mice were less in comparison to the FDG mice.

CD^+ cells. In the FG mice, the splenic $CD4^+$ cells and $CD4^+/CD8^+$ were significantly increased compared to CG mice, and in the FDG mice were significantly increased compared to DG mice (Table III).

VEGF expression. The protein expression of VEGF significantly downregulated in carcinoma of the FNJD mice compared to the CG mice. The expression of VEGF was significantly reduced in the FDG mice than DG mice (Fig. 2).

Discussion

Angiogenesis in tumors was described by Folkman (14), and it was proposed that tumor growth and metastasis are angiogenesis-dependent. It is now widely accepted that tumor-angiogenesis plays a crucial role in tumor growth and metastasis formation. Among several angiogenic activators, VEGF and its receptors represent one of the major inducers of tumor angiogenesis. Thus, this system has become the focus of therapeutic interventions.

TCM has been confirmed to effectively reduce toxic side effects and enhance curative effects of chemotherapy, palliate clinical syndrome, prevent recurrence and metastasis, and improve quality of life and immune function (15,16). The study guided by TCM theory believes that disharmony of Qi function (according to the fundamental theory of TCM, Qi is often translated as vital energy) is crucial to lung cancer. The herbs in the compound formula, FJND, for regulating Qi flow in the lungs, as well as for anticancer, played the roles of assistant ingredients. Ginsenosides, belonging to dammaranes, beneficially target the inhibition of tumor angiogenesis by suppressing its inducer in the endothelial cells of blood vessels and prevent adhering, invasion and metastasis of tumor cells (17). Polysaccharides from the mycelia of Indian buead also showed antitumor effects (18). An ethanol extract of *Scutellaria baicalensis* inhibited the growth of tumor cells, arrested the cell cycle, induced cell apoptosis and increased the content of tumor necrosis factor-α in serum (19). *Hedyotis Diffusa* Willd extract induces apoptosis via activation of the mitochondrion-dependent pathway in human colon carcinoma cells (20-22). *Rhizoma Curcumae* is a popular type of TCM whose essential oils are widely used in the treatment of cancer in China. Elemene, one of the chemical compositions of *Rhizoma Curcumae*, has already been approved by China's State Food and Drug Administration as an anticancer adjuvant drug and has been prescribed as a part of certain cancer treatment regimens in China. Another ingredient, furanodiene, significantly inhibits the proliferation of human umbilical vascular endothelial cells and inhibits VEGF-induced proliferation (23,24).

Chai Hu Long Gu Mu Li soup is a commonly used formula in the treatment of diseases of the neuropsychological system (such as chest congestion and anxiety). Therefore, FJND from Chai Hu Long Gu Mu Li soup, the herbs of which have the function of regulating Qi flow of the lungs, played the role of the sovereign ingredient. The effect of mood and depression on immunity has been widely discussed. Cancer patients frequently suffer from psychological comorbidities, such as depression and elevated perceived stress (25-28).

The results show that FJND can improve spontaneous activity of carcinoma-bearing mice. The present study analysed $CD4^+$ and $CD8^+$ in carcinoma-bearing mice receiving FJND therapy during chemotherapy. Compared to the DG mice, the level of $CD4^+$ and $CD4^+/CD8^+$ in FDG mice increased significantly. T cells are also key members of adaptive immunity against tumorigenesis. VEGF solid tumor secreted through autocrine and paracrine prevents the expression and secretion of cytokines (interleukins) and suppresses immune cell proliferation. Thus, tumor cells escape from the immune surveillance of the tumor-bearing host (29).

Therefore, these findings suggest that the antitumor mechanism of FJND may be due to the inhibition expression of the VEGF protein and the recovery of the immune balance.

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