Original Experimental Research 实验论著

Effects of Feiyanning Decoction on proliferation of lung adenocarcinoma cell line and their production of interleukin-6 and interleukin-8 induced by tumor necrosis factor-α

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Objective: To study the effects of Feiyanning Decoction, a compound traditional Chinese herbal medicine, on proliferation of lung adenocarcinoma cell line A549 cells and their production of interleukin-6 (IL-6) and IL-8 induced by tumor necrosis factor-α (TNF-α).
Methods: A549 cells were incubated with rat serum containing Feiyanning Decoction at 15% for 24, 48 and 72 h respectively. The cell proliferation was examined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt assay (WST-8). The production of IL-6 and IL-8 was tested by enzyme-linked immunosorbent assay after 48-hour treatment of reagents, and the expressions of IL-6 and IL-8 mRNAs were detected by reverse transcription-polymerase chain reaction.
Results: Serum containing Feiyanning Decoction had obvious inhibitive functions in A549 cell proliferation after 48- and 72-treatment. TNF-α (1 μg/L) strongly induced the production of IL-6 and IL-8 as compared with the control serum in A549 cells, and the induced cytokine production was significantly suppressed by 15% serum containing Feiyanning Decoction ($P<0.01$). In addition, serum containing Feiyanning Decoction could inhibit the mRNA expressions of IL-6 and IL-8 ($P<0.01$).
Conclusion: Feiyanning Decoction can inhibit IL-6 and IL-8 production induced by TNF-α. It is therefore expected to be a new strategy for treating lung cancer.

Keywords: Feiyanning Decoction; cytokine; lung neoplasms; serologic pharmacology; *in vitro*

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肺岩宁方对人肺癌细胞系增殖及肿瘤坏死因子α诱导的白细胞介素6和白细胞介素8产物的影响

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目的：探讨中药肺岩宁方含药血清对人肺癌细胞系 A549 细胞增殖及肿瘤坏死因子 α(tumor necrosis factor-α，TNF-α)诱导的 A549 细胞分泌白细胞介素 6(interleukin-6，IL-6)和 IL-8 的影响。
方法：肺岩宁方含药血清培养 A549 细胞 24、48 和 72 h 后，采用四唑单钠盐法检测肺岩宁方含药血清对 A549 细胞增殖的影响；肺岩宁方含药血清培养 A549 细胞 48 h 后，酶联免疫吸附测定法检测肺岩宁方含药血清对 TNF-α 诱导的 IL-6 和 IL-8 分泌的影响，逆转录聚合酶链式反应检测 IL-6 和 IL-8 mRNA 表达水平的变化。
结果：肺岩宁方含药血清对 A549 细胞增殖具有明显的抑制作用。1 μg/L TNF-α 可明显提高 A549 细胞上

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清液中 IL-6 和 IL-8 的分泌水平，肺岩宁方含药血清与 TNF-α 共同作用后，可抑制 IL-6 和 IL-8 的分泌（$P<0.01$）及 IL-6、IL-8 mRNA 的表达（$P<0.01$）。

结论：肺岩宁方含药血清对 TNFα 诱导 A549 细胞分泌 IL-6 和 IL-8 具有明显的抑制作用，这为其临床治疗肺癌提供了一定的实验依据。

关键词：肺岩宁；细胞因子；肺肿瘤；含药血清；体外研究

A causal relationship between inflammation and cancer has been suspected. Rudolf Virchow demonstrated the presence of leukocytes in malignant tissues and claimed that tumors arise from regions of chronic inflammation[1]. There is also increasing recognition that inflammatory cells, chemokines, and cytokines are implicated in tumor cell motility and invasive potential and metastases. Indeed, tumor biopsies indicated that inflammatory mediators were present in the tissue microenvironment of almost all solid tumors[2]. The microenvironment in and around tumors contains cells of the innate immune system that secrete pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-8[3]. This environment enhances cell proliferation, survival, migration, as well as angiogenesis, thereby promoting tumor development. Nuclear factor kappaB (NF-κB) is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens, and is a key regulator in the inflammatory response to infection[4]. The activation of NF-κB has been shown to play an important role in enhancing the expressions of several inflammatory cytokine genes, including TNF-α, IL-6 and IL-8. That phenomenon is observed in various cell types upon stimulation with such agonists as IL-1 and TNF-α. Feiyanning Decoction is a compound traditional Chinese herbal medicine for nourishing qi and yin and diminishing stagnation by detoxification functions, which could prevent invasion and metastasis of lung cancer[5, 6]. Recently, we have reported that serum containing Feiyanning Decoction could inhibit NF-κB activation induced by TNF-α[7]. The purpose of this present study was to further examine the effects of serum containing Feiyanning Decoction on production of IL-6 and IL-8 activated by TNF-α in A549 cells by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) methods.

1 Materials and methods

1.1 Materials Feiyanning Decoction [*Radix Astragali Mongolici* (Sheng Huangqi) 40 g, *Rhizoma Atractylodis Macrocephalae* (Baizhu) 15 g, *Succys Bufo* skin (Ganchanpi) 9 g, *Nidus Vespae* (Fengfang) 9 g, *Rhizoma Paridis* (Qiye Yizhihua) 15 g, *Rhizoma Polygonati Sibirici* (Huangjing) 30 g, *Herba Epimedii Brevicornus* (Xianlingpi) 15 g, *Ganoderma Lucidum* (Lingzhi) 30 g, *etc*] was provided by Longhua Hospital Pharmacy, Shanghai University of Traditional Chinese Medicine. The above drugs were soaked in 2 000 mL distilled water (DW) for 2 h, boiled for 60 min for the first decoction; the second decoction was added DW to 1 000 mL, boiled for 60 min. After mixed, the Feiyanning Decoction was condensed with water bath to crude drug content 2.0 g/mL and stored at 4 ℃ until use. Fetal bovine serum (FBS) was purchased from Shanghai Huamei Biotechnology Company. RPMI 1640 medium and 0.25% trypsin were purchased from Gibco Company, USA. Recombinant TNF-α was purchased from Calbiochem, Germany. Dimethyl sulfoxide (DMSO) was purchased from Santa Cruz, USA. IL-6 and IL-8 ELISA kits were purchased from Pierce Chemical, Rockford, USA.

1.2 Methods

1.2.1 Preparation of serum containing Feiyanning Decoction A total of 16 male Wistar rats were obtained from Shanghai Slac Laboratory Animal Co., Ltd. with body weight (300 ± 20) g and permit number was SCXK (Hu) 2007-0005. They were randomly divided into two groups (8 animals per group). Rats in treatment group were administered with Feiyanning Decoction at a dose of 22 mL/kg daily (equivalent to the human clinical dose of 8 times) based on body weight[8]; rats in control group were administered with equal volume of normal saline twice daily. According to serum preparation programs[9], rats were administered drugs daily by oral gavage for 3 days. The sera of same group were mixed, inactivated for 30 min in a 56 ℃ water baths, and then filter-sterilized through a 0.22 μm membrane filter. The sera were stored at −20 ℃ until use.

1.2.2 Cell culture The human lung cancer cell line A549 was originally obtained from the American Type Culture Collection (ATCC) and maintained in 5% CO_2 at 37 ℃ in RPMI 1640 medium containing 10% FBS. The A549 cells were divided into four groups: control serum group (serum culture medium), TNF-α group (TNF-α 1 μg/L), serum containing Feiyanning group (15% serum containing Feiyanning) and integrated group (TNF-α 1 μg/L plus 15% serum containing Feiyanning).

1.2.3 Cell proliferation detected by WST-8 assay The cell proliferation was evaluated by using WST-8 assay. A549 cells (1×10^4 cells/well) were seeded into 96-well plates in 100 μL of culture medium overnight, and then treated with corresponding serum. At the indicated times (24, 48 and

72 h), 10 μL of WST-8 reagent solution (Cell Counting kit, Dojindo Laboratories, Japan) was added and incubated for 2 h. Optical density (OD) value was detected at a test wavelength of 450 nm according to manufacturer's instructions. Percentage of inhibition of proliferation was calculated as follows: Percentage of inhibition = (mean control OD − mean experimental OD)/mean control OD × 100%.

1.2.4 Production of IL-6 and IL-8 detected by ELISA After treatment for 48 h, IL-6 and IL-8 concentrations in the supernatants were measured by using commercial ELISA kits (Pierce Chemical, Rockford, IL, USA), according to the manufacturer's instructions. Values were expressed as pg per milligram protein (pg/mg prot).

1.2.5 Total RNA extraction Total RNA was extracted from A549 cells by using TRIzol kit according to the manufacturer's instructions. The concentration and purity of total RNA obtained were detected by spectrophotometric measurements at 260 and 280 nm.

1.2.6 IL-6 and IL-8 mRNA expressions detected by RT-PCR IL-6 and IL-8 mRNA expressions in A549 cells were determined by RT-PCR. The primers used were presented in Table 1. The reaction parameters were as follows: 94 ℃ 5 min, 94 ℃ 1 min, 55 ℃ 1 min, 72 ℃ 2 min for 28 − 35 cycles and 72 ℃ extension for 10 min. The PCR products were separated in 2% agarose gel, visualized by staining with ethidium bromide and analyzed with Image 1.62.

1.3 Statistical analysis Data were presented as $\bar{x} \pm s$. Statistical analysis was performed with SPSS 10.0 statistical software. Comparisons among groups were performed with one way analysis of variance or factorial design analysis of variance.

2 Results

2.1 Growth inhibition of A549 cell line Compared with control serum, the proliferation of the A549 cells was inhibited after treated with serum containing Feiyanning Decoction at 48 h and 72 h (Table 2).

2.2 Effects of serum containing Feiyanning Decoction on production levels of IL-6 and IL-8 Compared with the control serum, there were no significant differences in IL-6 ($F = 2.695$, $P = 0.352$, Figure 1) and IL-8 levels ($F = 3.395$, $P = 0.372$, Figure 1) treated with 15% of serum containing Feiyanning Decoction; TNF-α strongly induced IL-6 ($F = 14.052$, $P = 0.004$) and IL-8 production ($F = 13.152$, $P = 0.005$) by approximately 2-fold. However, the induced production of IL-6 and IL-8 was significantly suppressed by 15% serum containing Feiyanning Decoction ($F = 15.025$, $P = 0.005$, $F = 14.421$, $P = 0.005$. Figure 1).

2.3 Effects of serum containing Feiyanning Decoction on expressions of IL-6 and IL-8 mRNAs Compared with the control serum, TNF-α strongly induced IL-6 ($F = 18.152$, $P = 0.004$) and IL-8 mRNA expressions ($F = 16.322$, $P = 0.005$). However, the induced IL-6 ($F = 11.047$, $P = 0.008$) and IL-8 mRNA expressions ($F = 12.631$, $P = 0.007$) were significantly suppressed by serum containing Feiyanning Decoction (Figure 2).

Table 1 Primers used for RT-PCR analysis

Gene	Primer sequence	Size of PCR product (bp)
IL-6	Forward: 5′-GATGCTACCAAACTGGATATAATC-3′	269
	Reverse: 5′-GGTCCTTAGCCACTCCTTCTGTG-3′	
IL-8	Forward: 5′-CTGTGTGAAGCTGCAGTTCT-3′	180
	Reverse: 5′-TAGGCAGACCTCGTTTCCAT-3′	
β-actin	Forward: 5′-AAGTACTCCGTGTGGATCGG-3′	547
	Reverse: 5′-TCAAGTTGGGGGACAAAAAG-3′	

Table 2 Effects of serum containing Feiyanning Decoction on proliferation of A549 cells

($\bar{x} \pm s$)

Group	n	OD value			Rate of inhibition after treatment (%)		
		24 h	48 h	72 h	24 h	48 h	72 h
Control serum	4	0.325±0.014	0.497±0.021	0.521±0.019	0	0	0
Serum containing Feiyanning Decoction	4	0.344±0.028	0.359±0.020	0.321±0.022	0.05±0.62	30.85±1.62*	37.66±1.48**

* $P<0.05$, ** $P<0.01$, *vs* control serum group.

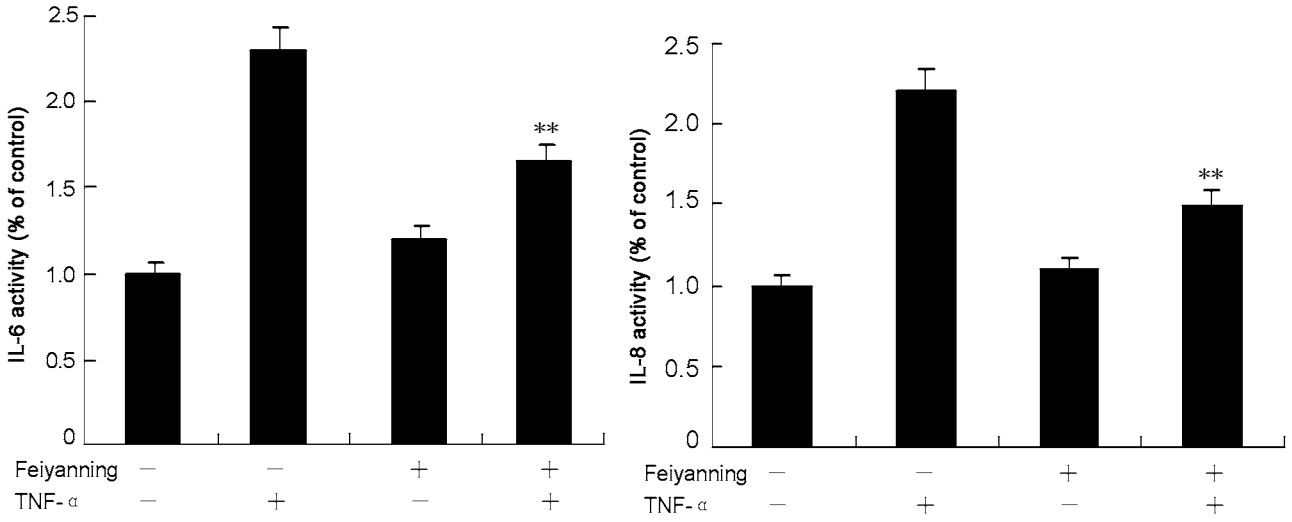

Figure 1 Effects of serum containing Feiyanning Decoction on IL-6 and IL-8 levels induced by TNF-α in A549 cells

Confluent cells were incubated for 48 h with or without 15% serum containing Feiyanning Decoction, or TNF-α (1 μg/L). Concentrations of IL-6 and IL-8 in supernatant were measured by ELISA. Bars represent $\bar{x} \pm s$ ($n = 3$). ** $P < 0.01$, *vs* TNF-α.

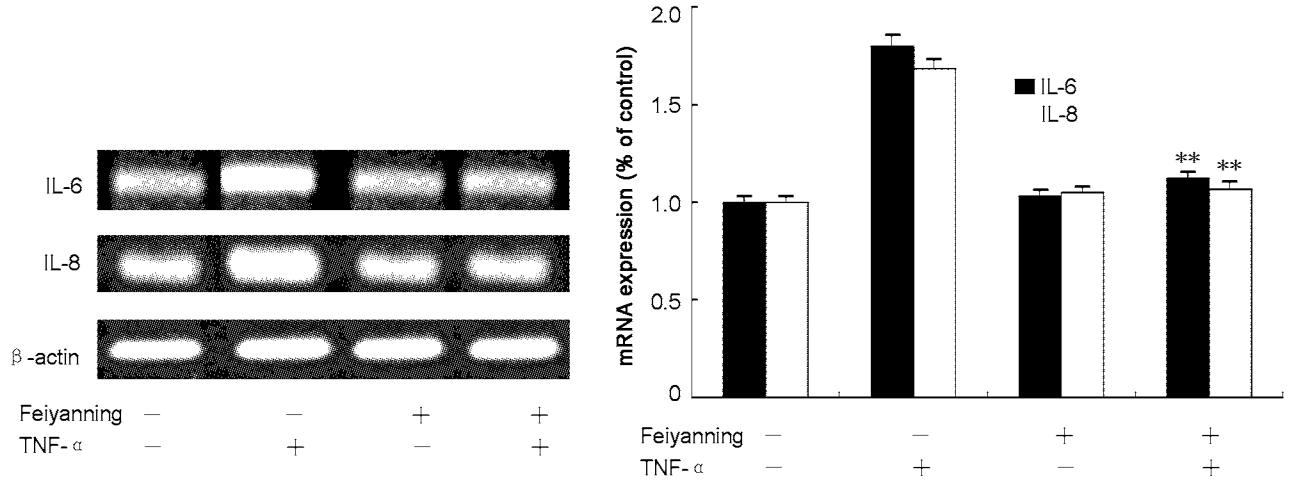

Figure 2 Effects of serum containing Feiyanning Decoction on IL-6 and IL-8 mRNA expressions in A549 cells

After the treatment, RNA was extracted. Levels of cytokines and β-actin mRNAs were examined by RT-PCR. The three panels on left show the expression levels of IL-6, IL-8 and β-actin mRNAs; bar graphs at the right show the expression levels of IL-6 or IL-8 relative to those of β-actin. The experiments were performed in triplicate. The data were represented as $\bar{x} \pm s$ ($n = 3$). ** $P < 0.01$, *vs* TNF-α.

3 Discussion

In the tumor microenvironment, inflammatory cells and molecules influence almost every aspect of cancer progress, including the tumor cells' ability to metastasize[10]. More importantly, proliferation in the setting of chronic inflammation predisposes humans to carcinoma of the large bowel, lung, liver, breast, urinary bladder, gastric mucosa, prostate, ovary, and skin[2]. The mixture of cytokines that is produced in the tumor microenvironment has an important role in cancer pathogenesis. Cytokines are released in response to infection, inflammation and immunity, which can inhibit tumor development and progression. Alternatively, cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. A group of cytokine proteins, including IL-1, IL-6, TNF and receptor activator of NF-κB ligand (RANKL), activate inflammation and are known to augment tumor cells' ability to metastasize by affecting several steps in the cells' dissemination and implantation at secondary sites[11]. Inflammatory cytokines lie downstream of the 'master' gene transcription factor for promoting inflammation – NF-κB – which is itself activated by them[12]. From clinical serum specimens, it was observed that MCP-1 and IL-8 levels were elevated in patients with localized lung cancer as compared with those in healthy donors[13].

Feiyanning Decoction is a compound traditional Chinese herbal medicine for nourishing qi and yin and diminishing stagnation by detoxification functions, which can prevent invasion and metastasis of lung cancer. Clinical studies showed that Feiyanning Decoction had functions of stabilizing advanced

lung cancer tumor, preventing tumor invasion and metastasis and improving the quality of life and survival time of patients[5, 6]. Chinese medicine serum pharmacology methods provided a reliable guarantee for studying complex components of Chinese medicine *in vitro*. Our data showed that 15% of serum containing Feiyanning Decoction could markedly inhibit A549 cell proliferation.

Serum levels of cytokines have been reported to be elevated in patients with breast, colorectal and lung cancers[14, 15]. IL-6, which has diverse biological effects on immune and inflammatory responses, is produced in response to an infection or an injury in a variety of cells, including monocytes, lymphocytes, fibroblasts, endothelial cells, and keratinocytes. The activation of NF-κB has been shown to play an important role in enhancing IL-6 expression[11]. Experimental studies showed that Feiyanning Decoction could inhibit NF-κB activity in A549 cells[7]. To evaluate the effects of Feiyanning Decoction on the production of the inflammatory markers, we measured the levels of IL-6 and IL-8 proteins produced in the supernatant of A549 cells. The TNF-α-induced IL-6 and IL-8 production was suppressed by serum containing Feiyanning Decoction. Our findings demonstrated that Feiyanning Decoction could not only inhibit the activation of NF-κB, but also decrease the expression levels of the pro-inflammatory cytokines (IL-6 and IL-8), which may influence the evolution of the human lung cancer inflammatory process.

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