



RAPID COMMUNICATION

Effects of a Shuangling Fuzheng anticancer preparation on the proliferation of SGC-7901 cells and immune function in a cyclophosphamide-treated murine model

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Abstract

AIM: To study the inhibitory effects of a Shuangling Fuzheng anticancer preparation (SFAP) on the human gastric cancer cell line SGC-7901 *in vitro* as well as its immune-modulated effects in a cyclophosphamide-treated murine model.

METHODS: MTT experiments and immunocytochemistry ABC experiments were performed for detecting the proliferation of SGC-7901 cells *in vitro* and protein expression of c-myc. The staphylococcal protein A (SPA) rosette test was utilized for measuring the ratio of T-lymphocyte subsets from peripheral blood in a cyclophosphamide-treated murine model. Enzyme-linked immunosorbent assay (ELISA) was performed for measuring the levels of serum sIL-2R in treated mice, while immunoturbidimetry was used for measuring the levels of immunoglobulins (Ig).

RESULTS: SFAP (40-640 mg/L, 48 h) inhibited the proliferation of SGC-7901 cells, and a positive correlation was noted between inhibitory effects and dosage. At a dosage of 160-320 mg/L in cultured cells, the expression of c-myc was decreased. SFAP (50-200 mg/kg) increased the percentage of CD3⁺ and CD4⁺ T-lymphocytes, the ratio of CD4/CD8, and the contents of Ig such as IgM, IgG or IgA, but decreased the levels of serum sIL-2R in peripheral blood from cyclophosphamide-treated mice.

CONCLUSION: SFAP can inhibit the proliferation of SGC-7901 cells *via* the c-myc gene. In addition, SFAP can modulate the cellular and humoral immunity in cyclophosphamide-induced immunosuppressed mice.

INTRODUCTION

Shuangling Fuzheng anticancer preparation (SFAP) is made through the basic modification of Fuzheng anticancer granules (FAG). Clinical research has shown that FAG have a therapeutic effect for many tumor patients. An empirical study has shown that FAG can prolong the life of tumor-bearing mice and inhibit transplanted tumors. However, this inhibition ratio is lower than 30%^[1-3]. Modern pharmacologic studies have shown that atracylodes macrocephalae volatile oil^[4], coixenolide^[5-7], polysaccharides^[8,9] and some other active constituents have anticancer activities. At the precondition of Chinese Medical Syndrome Differentiation, we extracted some of the effective anticancer constituents from FAG and developed SFAP. The main of making SFAP include extraction of volatile oils from atracylodes macrocephalae, coixenolide from coix seed, and polysaccharides from part of Fuzheng traditional Chinese medicines, boiling the other drugs with water as per the traditional method, and concentrating all constituents into a capsule preparation. The results of this experiment showed that the ratio of SFAP anticancer activity was significantly greater than 30%^[10]. The present study reports the effects of SFAP on the proliferation of human gastric cancer cells and the expression of related genes *in vitro*, as well as its modulating effects on immune function in a cyclophosphamide-treated murine model.

MATERIALS AND METHODS

Cell line and animals

Human gastric cancer SGC-7901 cells, purchased from the Department of Cellular and Molecular Biology, Shanghai Institute of Biochemistry and Cell Biology, Academia

Sinica, were cultured *in vitro* for 2-3 d. ICR mice (at the age of 6-7 wk, weighing 18-22 g; 50% females) were obtained from Yangzhou University Medical Centre [animal production license number: SCXK (Su) 2002-0009].

Drugs and reagents

SFAP was made from *Smilaxis rhizome*, Tuckahoe, honey-fried *Astragalus mongholicus*, *Atractylodes macrocephala*, coix seed, processed *Pinelliae tuber*, *Aurantii nobilis pericarpium* and the like. We extracted volatile oil from *Atractylodes macrocephala*, coixenolide from *Coicis semen*, and polysaccharides from other drugs by boiling in water. They are then mixed at the concentrations specified according to previous formulations. Lastly, the dried drugs were ground into coffee granules. An invention patent for the SFAP preparation protocol has been applied (application No.: 200610161699.5). SFAP was resuspended with RPMI 1640 (GibcoBRL, Maryland, USA) to a suitable dosage, filtrated, sterilized and stored at 4°C for testing *in vitro*. MTT was from Sigma (St. Louis, MO, USA). Calf serum, trypsin (1:250), ABC immunohistochemical detection kit (concentrated type) and DAB substrate kit were from Huamei Biotechnology Company. Monoclonal mouse anti-human c-myc antibodies were provided by Huamei Biotechnology Company. Red blood cell rosette reagent used to restrain the monoclonal antibody hypersusceptibility to CD3, CD4 or CD8 from treated mice was from the Wuhan Biologic Product Institute. The soluble interleukin-2 receptor (sIL-2R) quantitative EIA kit was from Shanghai Senxiong Technology Industry Limited Company. Ig kit was obtained from Shanghai Sun Biological Products Co. Ltd. Injectable cyclophosphamide (CTX, batch number: 20051204) was obtained from Shanxi Jinde Medicine Limited Company. Ultrapure water was self-prepared. Other reagents were all analytical reagents made in China. Adriamycin was from Shenzhen Wanle Pharmaceutical Co. Ltd.

MTT experiment

SGC-7901 cells growing exponentially were digested with 2.5 g/L trypsin, then washed several times with PBS (pH 7.2). They were then counting and RPMI 1640 contained 100 mL/L newborn bovine serum medium in order to adjust cell density to 1×10^8 cells/L. After the final cell suspensions were adjusted the to 100 μ L/well, 96-well plates were put into an incubator that containing 50 mL/L CO₂, at 37°C for 24 h. Then, 100 μ L RPMI 1640 containing different concentrations of SFAP (40-640 mg/L) was added to each well. Cells were cultured for 48 h. Four hours before the end of culture, 100 μ L supernatant was removed and 10 μ L MTT (the final concentration was 5 g/L) was added and cultured for 4 h, to which acid isopropyl alcohol was subsequently added per routine. The absorbance (A) for each well were measured at 570 nm with an ELISA reader. The inhibition ratios were calculated with the following formula: $(1 - \frac{\text{the mean of treated group}}{\text{the mean of control group}}) \times 100$.

Immunohistochemical detection

Cells were cultured as previously described and the cell density was adjusted to 2×10^8 cells/L. After the above-mentioned cell suspensions were added to a 4 mL culture

flask, the flask was placed into an incubator containing 50 mL/L CO₂ at 37°C for 24 h. Then, culture flasks were randomly divided into groups for different concentrations of SFAP (4 mL), and in the control group RPMI 1640 (4 mL) was added. After continuous culture for 48 h, cells were isolated, digested, and washed. The cells were fixed with 40 g/L formalin, imbedded with paraffin, and then consecutively sliced at a thickness of 3 μ m. The paraffin slices were dewaxed and washed with graded alcohol, 3 mL/L H₂O₂ methanol, and incubated for 10 min at room temperature in order to inactivate the endogenous peroxidase activity and eliminate non-specific staining. Next, mouse anti-human c-myc monoclonal antibody attenuated at 1:100 was added, and incubated at 4°C following the addition of biotinylated sheep anti-mouse IgG. After the above-mentioned mixture was incubated at 37°C in a moisture-saturated plastic chamber for 30 min, ABC compound was added and incubation was continued for 30 min, followed by three 5-min PBS washes. Staining with DAB was observed by microscope for 5-10 min, after which the cells were washed with tap water for 3 min in order to terminate the reaction. Finally, the slices were stained with haematoxylin, dehydrated, cleared, mounted and observed with a microscope. A light yellow cytolymph indicated positive expression of c-myc protein. Every slice was observed under five high power fields and each positive expression ratio was counted per the following formula: positive expression ratio = $(\frac{\text{the sum of positive cells}}{\text{the sum of all the cells}}) \times 100\%$.

Influence on of SFAP-lymphocyte subsets from the peripheral blood

The animal study protocol was approved by the Ethics Committee of Yangzhou University Medical College.

A total of 30 ICR mice, half being female, were randomly divided into five groups: NS group, CTX group and three (CTX + SFAP) treatment groups. The ICR mice in the NS group were treated *ig* and *ip* with 0.01 mL/g normal saline (NS) once a day for 5 d; the CTX group was treated *ip* with 40 mg/kg CTX and *ig* with NS once a day for 5 d; three (CTX + SFAP) treatment groups were treated *ip* with CTX 40 mg/kg and *ig* with SFAP, such that the dose was 50, 100 or 200 mg/kg SFAP once a day for 5 d. The Staphylococcal protein A (SPA) rosette test was utilized on the sixth day for measuring the ratio of T-lymphocyte subsets from peripheral blood. The eyes of the mice were removed and venous blood was gathered (about 1 mL), which was then placed into an anticoagulated heparin test tube. One milliliter of NS was added to the test tube. After mixing, 2 mL of T-lymphocyte cell separating medium was added, and then the blood sample was centrifuged for 15 min at 3000 r/min. The intermediate buffy coat was collected and washed three times with Hank's solution. The number of T-lymphocytes was then adjusted to 1000/mm³. The intermediate lymphocyte suspension (10 μ L) was placed into three test tubes and 10 μ L of the red blood cell suspension was added to each tube in order to restrain monoclonal antibody hypersusceptibility of CD3, CD4 or CD8 in mice. After incubating the above-mentioned suspension at 37°C for 30 min, it was centrifuged for

Table 1 Inhibitory effect of SFAP on proliferation of SGC-7901 cells (mean \pm SD)

Group	Dose (mg/L)	A value	Rate Inhibition (%)
Control	0	0.290 \pm 0.027	0
SFAP	40	0.248 \pm 0.055	14.5
SFAP	80	0.239 \pm 0.044	17.6
SFAP	160	0.230 \pm 0.034	20.7
SFAP	320	0.220 \pm 0.041 ^a	24.1
SFAP	640	0.212 \pm 0.009 ^b	26.9
Adriamycin	2	0.125 \pm 0.037 ^b	56.9

^a*P* < 0.05, ^b*P* < 0.01 *vs* Control.**Table 2** Influence of SFAP on expression of *c-myc* protein in SGC-7901 cells (mean \pm SD)

Group	Dose (mg/L)	Positive <i>c-myc</i> rate (%)
Control	0	22 \pm 6
SFAP	160	11 \pm 3 ^b
SFAP	320	9 \pm 3 ^b

^b*P* < 0.01 *vs* control.

3 min at 1000 r/min, made into a slice, dried, and was colored with Gliemsa solution for 30 min. The number of cells positive for CD3, CD4 or CD8 were then observed through a microscope. A T-lymphocytes binding with more than 3 red cells (rosette formation) was defined as positive. More than 200 T-lymphocyte were counted, and the percentage of cells with rosette formation was calculated.

Influence on serum sIL-2R contents

As described above, ICR mice were grouped and treated; in this case, the dose of CTX administered was 10 mg/kg. The eyes were removed, venous blood was gathered (about 1 mL) and blood serum was centrifuged on the sixth day. Per kit instructions, ELISA was used to measure the levels of sIL-2R from blood serum in treated mice. The sample dilution (50 μ L) and the solution awaiting detection (50 μ L) were put into each well of the reaction plate. After mixing, the reaction plate was stored at 37°C for 2 h, and was subsequently washed 4–6 times and blotted on filter paper. The first antibody (100 μ L) was placed into each well, the reaction plate was stored at 37°C for 1 h, and then washed as described above. The enzyme-labeled antibody (100 μ L) was placed in each well and was stored at 37°C for 1 h; the reaction plate was then washed as described above. The substrate (100 μ L) was placed into each well, and was allowed to react for 5–10 min in the dark at 37°C. Lastly, one drop of stop buffer was put in each well and mixed, from which absorption was then detected at 492 nm. The levels of sIL-2R were determined according to the standard curve of absorption-concentration.

Influence on serum Ig contents

As described above, the dose of CTX was changed to 10 mg/kg. Immunoturbidimetry was utilized for measuring the levels of Ig from blood serum in mice on the sixth

Table 3 Influence of SFAP on T-lymphocyte subsets from peripheral blood in cyclophosphamide-treated mice

Group	Dose (mg/kg)	CD3 (%)	CD4 (%)	CD8 (%)	CD4/CD8
NS control	0	46.2 \pm 4.5	28.5 \pm 1.4	17.7 \pm 2.16	1.6 \pm 0.1
CTX control	40	37.5 \pm 2.4 ^b	21.5 \pm 2.2 ^b	16.3 \pm 1.6	1.3 \pm 0.1 ^b
SFAP + CTX	50 + 40	38.7 \pm 2.2	22.2 \pm 2.3	16.7 \pm 2.0	1.3 \pm 0.2
SFAP + CTX	100 + 40	39.7 \pm 3.1	24.2 \pm 2.3	16.2 \pm 1.5	1.5 \pm 0.1 ^c
SFAP + CTX	200 + 40	42.3 \pm 2.5 ^d	26.7 \pm 2.3 ^d	16.8 \pm 1.5	1.6 \pm 0.1 ^d

^b*P* < 0.01 *vs* NS control; ^c*P* < 0.05, ^d*P* < 0.01 *vs* CTX control group.

day. The eyes were removed, venous blood was collected (1 mL), and the sample was centrifuged. The diluted blood serum (40 μ L, 10 μ L or 80 μ L) was put into a test tube, and 1 mL IgA, IgG or IgM was added to each tube. After mixing, the samples were immersed in a water bath at 37°C for 15 min, a semi-automatic biochemistry analyzer zero was adjusted to 340 nm with NS, and the absorption for each test was detected. The levels of IgA, IgG or IgM were computed according to the absorption-concentration standard curve.

Statistical analysis

Data are presented as the mean \pm SD for all experiments. Comparisons of numerical data were performed using the *t* test, and comparisons of categorical data were performed using χ^2 test.

RESULTS

Effect of SFAP on proliferation of SGC-7901 cells

SFAP (40–640 mg/L, 48 h) inhibited proliferation of SGC-7901 cells in a dose-dependent manner. This effect was statistically significant at dosages of 160 mg/L and 320 mg/L (Table 1).

Effect of SFAP on *c-myc* protein expression in SGC-7901 cells

SFAP decreased the expression of *c-myc* protein in SGC-7901. This was statistically significant when compared with the control group (Table 2).

Effect of SFAP on distribution of T-lymphocyte subsets from the peripheral blood in mice

CTX (40 mg/kg) decreased the percentage of CD3⁺ and CD4⁺ T-lymphocytes and the ratio of CD4/CD8 from the peripheral blood, which was statistically significant compared with the control group. The percentage of the CD3⁺ and CD4⁺ T-lymphocytes was increased in all three SFAP dosage groups (50, 100 or 200 mg/kg), with the difference being statistically significant in the largest dosage group (200 mg/kg), compared with the CTX group. The moderate and largest dosage groups (100 and 200 mg/kg, respectively) increased the ratio of CD4/CD8 from the peripheral blood, and this was statistically significant compared with the CTX group (Table 3).

Effect of SFAP on serum sIL-2R and Ig contents

CTX (10 mg/kg) increased the levels of sIL-2R in the serum, which was highly significantly different compared with the NS control group (*P* < 0.01). Both moderate and largest dosage

Table 4 Influence of SFAP on serum sIL-2R and Ig levels in cyclophosphamide-treated mice (mean \pm SD)

Group	Dose(mg/kg)	sIL-2R (pmol/L)	IgA(g/L)	IgG(g/L)	IgM(g/L)
NS control	0	30.4 \pm 2.5	2.92 \pm 0.84	8.07 \pm 0.94	2.31 \pm 0.51
CTX control	10	77.8 \pm 8.5 ^b	1.77 \pm 0.14 ^b	6.35 \pm 0.63 ^b	1.68 \pm 0.14 ^a
SFAP + CTX	50 + 10	75.9 \pm 6.4	2.02 \pm 0.31	6.87 \pm 0.34	1.82 \pm 0.27
SFAP + CTX	100 + 10	59.6 \pm 6.1 ^d	2.31 \pm 0.48 ^c	7.70 \pm 0.37 ^d	2.16 \pm 0.33 ^d
SFAP + CTX	200 + 10	52.7 \pm 5.7 ^d	3.11 \pm 0.72 ^d	7.87 \pm 0.59 ^d	2.49 \pm 0.46 ^d

^a $P < 0.05$, ^b $P < 0.01$ vs NS control; ^c $P < 0.05$, ^d $P < 0.01$ vs CTX control.

groups (100 mg/kg and 200 mg/kg) decreased the levels of sIL-2R in serum from mice treated with cyclophosphamide, which was statistically significant compared with the CTX group (Table 4). In addition, CTX (10 mg/kg) inhibited the elaboration of Ig, and was significantly different compared with the NS control group. All three SFAP groups increased the levels of IgA, IgG and IgM in the serum when induced by cyclophosphamide, with statistical significance for the large and moderate dose groups when compared with the CTX control group (Table 4).

DISCUSSION

The target cells in this study were from the cell line SGC-7901. After culturing *in vitro* for 48 h, MTT experiments were utilized to detect SGC-7901 proliferation *in vitro*. The results proved that SFAP (40-640 mg/L) inhibited the proliferation of SGC-7901 and the correlation between the inhibitory effects and the dosage was positive, such that there was a dose-effect relationship. From this observation, we postulate that SFAP directly inhibits the proliferation of SGC-7901 cells. The c-myc protein is the key to controlling the proliferation of these cells, and has been shown to induce tumor cells into multiplication cycles and promote hyperplasia^[11-14]. We utilized the immunocytochemistry ABC experiment to detect the influence of SFAP on protein expression of c-myc in SGC-7901 cells. The results proved that SFAP (40-640 mg/L) decreased protein expression of c-myc in these cells. These data imply that c-myc probably affects the regulation of tumor cell proliferation. Considerable evidence has demonstrated that when immunomodulatory mechanisms are suppressed, the incidence rate of the tumor increases and the speed of cancer metastasis and growth quickens. When the malignancy grows constantly or radiotherapy and/or chemotherapy are applied to manage the tumor, the immunomodulatory mechanisms of the patient decline. Thus, there is a close relationship between immunomodulatory activity and the appearance of cancer^[15-18].

Cellular immunity is the key to host tumor immunity, and T-lymphocytes are the critical immune component for tumor immunity. The cluster differentiation (CD) antigen is an important immune cell membrane molecule. CD3 antigen is present on the cell membrane of all mature T-lymphocytes in the peripheral blood and is involved in CD4⁺ and CD8⁺ T-lymphocyte response. The former is mainly a helper T-lymphocyte, which can also assist B cells to generate antibodies, and the later is chiefly a suppressor T-lymphocyte which can release suppressive cytokines to

suppress cellular and humoral immune responses. When T-lymphocyte-mediated cellular immunity is weakened, CD4 is decreased while CD8 is increased, thus the ratio of CD4/CD8 decreases^[19-23]. Our experimental results show that SFAP treatment at all used dosages increased the percentage of CD4⁺ cells in the CTX mouse model, and at the same time increased the ratio of CD4/CD8. IL-2 is an important immune factor secreted by helper T-lymphocytes, which after combining with IL-2R could promote immune cell proliferation and suppress tumor cell division. sIL-2R derives mainly from active T-lymphocyte membrane receptor proteins which could competitively bind to IL-2, thereby restraining the biological activity of IL-2. Thus, sIL-2R is regarded as an important immune suppressor^[24-28]. The experimental results in the present study show that SFAP significantly decreased the level of sIL-2R in a CTX mouse model, which suggests that SFAP could enhance cellular immune function in mice inhibited by CTX.

Humoral immunity is critical to host tumor immunity^[29]. For example, a variety of antibodies from the blood promote effector cells to recognize and kill tumor cells. Our results show that SFAP is capable of enhancing humoral immune function in mice inhibited by CTX by increasing the serum levels of IgA, IgG and IgM.

Many traditional Chinese medicines derived from the Fuzheng drug are capable of enhancing immune function with few adverse effects; however, the amount of tumor death is small. Traditional cytotoxic drugs are powerful enough to kill tumor cells, but they come with high toxicity, especially with regards to immune and hemotologic function^[30]. Thus, many doctors avoid using these kinds of drugs to their patients. The results of our experiments show that SFAP directly inhibits the proliferation of SGC-7901 cells and enhances the cellular and humoral immune function inhibited by CTX. These effects may be beneficial for cancer prevention, anti-cancer therapies, prevention of cancer relapse and metastasis, and avoidance of adverse reactions from radiotherapy and chemotherapy.

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COMMENTS

Background

Malignant tumors are one of the most common causes of mortality in China and the world. While surgical treatment predominates in comprehensive therapy

for tumors, anti-tumor drugs still play an important role. Currently, cytotoxic compounds remain the major constituent of chemotherapy drugs. However, cytotoxic compounds have poor therapeutic effects on the solid tumors and generate higher toxic side-effects and drug resistance. Many Chinese traditional medicines can enhance the immune function of the host, with few toxic side-effects when used in the treatment of tumors.

Research frontiers

Shuangling Fuzheng anticancer preparation (SFAP) consists of Fuzheng and Quxie Chinese traditional medicines. SFAP directly inhibits the proliferation of cancer cells through the action of Fuzheng, and also promotes cellular and humoral immune function in a cyclophosphamide-treatment mouse model through its Quxie action.

Innovations and breakthroughs

Fuzheng anticancer granules (FAG) promote immune function and inhibit transplanted tumors in mice, but the inhibition rate is less than 30%. SFAP has the same constituents of FAG, but improves the action of FAG. At the precondition of Chinese Medical Syndrome Differentiation, the main characteristics of SFAP production were extraction of volatile oils from *Atractylodes macrocephalae*, coixenolide from coix seed, and polysaccharides from part of Fuzheng traditional Chinese medicines, boiling the other drugs with water as per the traditional method, and concentrating all constituents into a capsule preparation. The results of this experiment showed that the ratio of SFAP anticancer activity was significantly greater than 30%. Moreover, SFAP could directly inhibit the proliferation of the cancer cells *in vitro*, and also promote immune function in cyclophosphamide-treated immunosuppressive mice.

Applications

The immune function of the human body has a close association with the development of cancer. On the one hand, when immune function is suppressed, the incidence of cancer increases and the speed of cancer metastasis quickens. On the other hand, when the malignancy grows constantly *in vivo* or radiotherapy or chemotherapy is given to cancer patients, immune function declines as the state of illness worsens. SFAP could directly control tumor cell growth and adjust the balance of cellular and humoral immunity of human body under different conditions. It exerts not only Fuzheng action, but also Quxie action. SFAP may be beneficial for all cancer patients and of applications in cancer prevention, anticancer therapy with few adverse reactions.

Peer review

The Chinese traditional medicine constituents of SFAP are logical and scientific. Its preparation process has many salient features. The preparation exerts not only Fuzheng action, but also Quxie action. The inhibition ratio of SFAP on tumor is higher than common Chinese traditional medicine, and the toxic side-effects are less than those of conventional anti-tumor chemotherapy drugs. As a new compound for anti-tumor therapy, SFAP should be developed as soon as possible.

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