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A polysaccharide from *Salvia miltiorrhiza* Bunge improves immune function in gastric cancer rats

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ABSTRACT

A neutral polysaccharide fraction (SMPA) prepared from the roots of *Salvia miltiorrhiza* by DEAE-cellulose and Sephadex G-100 chromatography was tested for its immune enhancing function in *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG)-induced gastric cancer rats by intragastric administration. SMPA (200 mg/kg) treatment not only increased the body weight, but also improved the immune organ indices. Furthermore, studies of various immunological activities in gastric cancer rats revealed that SMPA significantly stimulated splenocyte proliferation, promoted anti-inflammatory cytokines (IL-2, IL-4 and IL-10) production, inhibited pro-inflammatory cytokine (IL-6 and TNF-α) secretion, augmented the killing activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTL), and increased phagocytotic function of macrophages in gastric cancer rats. In addition, SMPA administration evidently elevated total intracellular granzyme-B and IFN-γ levels produced by splenocytes in gastric cancer rats. Taken together, these results suggested that SMPA could act as an effective immunomodulator and might be explored as a potential supplemental source for gastric cancer therapy.

1. Introduction

Gastric cancer is the second most common cause of cancer-related deaths worldwide (Neugut, Hayek, & Howe, 1996). Surgery, chemotherapy, and radiotherapy are still the major conventional cancer therapies for this malignancy (Rugge, 2007). But these therapies have been ineffective in many cases due to their adverse reactions (Fennelly, 1995; Ross & Small, 2002) and multidrug resistance (MDR) of tumor cells to chemotherapeutic agents (Liscovitch & Lavie, 2002). Despite varied treatment strategies, the control of this cancer at an advanced stage remains problematic. Therefore, there is an urgent need for the development of novel therapeutic strategies to improve the life expectancy of gastric cancer patients.

To the best of our knowledge, immune suppression is clearly observed in cancer patients and tumor-bearing animals, suggesting that tumor cells impair the function of immune system (Vasievich & Huang, 2011). The low immune function of an organism in turn may not only result in the progression of a tumor, but may also be one of the most important factors that prevent the tumor patients' recovery. Effective immunity protects against pathogens, enables wound healing and tissue repair, and defends against some types of tumors. Tumor cells can evade immune detection and elimination by various mechanisms (Sephton et al., 2009). When this occurs, robust immunity is still essential for maintaining the general health of the cancer patient. The discovery and identification of new antitumor drugs, which can potentiate the immune function, has become an important goal of research in immunopharmacology and oncotherapy (Mitchell, 2003). In the last few decades, many polysaccharide–protein complexes with immunomodulatory activity from medicinal herbs have shown promise as potential anticancer (Schepetkin & Quinn, 2006). These polysaccharides usually have low toxicity and few side effects, which make them appropriate for immunotherapy against cancer (Liu et al., 2013; Zeng, Ju, Shen, Zhou, & Huang, 2006; Yang et al., 2013). The major mechanisms by which polysaccharides protect cells are generally thought to be through activating the host immune response.

The dried root of *Salvia miltiorrhiza* Bunge, referred to as 'Danshen', is one of the most commonly used medicinal herbs in China and is officially listed in the Chinese Pharmacopeia (De Palma et al., 2008), and has been used in traditional Chinese medicine (TCM) for more than 2000 years to prevent and treat various human diseases such as hepatitis, coronary artery disease, apoplexy, tumor

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growth and immunological disorders. In particular, it was found that tanshinones from this plant have been recently shown to possess some activities against various human cancer cells, such as prostate, lung, breast, leukemia, glioma, stomach, nasopharynx, and liver (Gong et al., 2011; Liu et al., 2013). However, to the best of our knowledge, no information has been reported on the anti-tumor activity of *S. miltiorrhiza polysaccharides* on gastric cancer in vivo, let alone its anti-cancer mechanism. *N*-Methyl-*N*′-nitro-nitrosoguanidine (MNNG) is a gastric carcinogen in several animal species and has been used in a number of systems to dissect the cocarcinogenic potential of various compounds in the induction of gastric adenocarcinoma (Ohgaki, Kleihues, & Sugimura, 1991; Zhang, Luo, Bi, & Zhou, 2012). Hence the objective of the present study is to prospectively examine the antitumor and immunomodulating activities of the polysacchrides isolated from *S. miltiorrhiza* in MNNG-induced gastric cancer rats and attempt to elucidate the mechanism behind it.

2. Materials and methods

2.1. Materials and chemicals

S. miltiorrhiza was obtained from Xi'an, China. DEAE-52 cellulose and Sephadex G-100 were purchased from Amersham Biosciences Co., Piscataway, NJ, USA; mannose (Man), ribose (Rib), rhamnose (Rham), glucuronic acid (GlcUA), galacturonic acid (GalUA), glucose (Glc), galactose (Gal), xylose (Xyl), arabinose (Ara), fucose (Fuc), trifluoroacetic acid (TFA), 1-phenyl-3-methyl-5-pyrazolone (PMP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS) were purchased from Sigma, Chemical Co., Saint Louis, Missouri, USA; RPMI-1640 medium, fetal calf serum (FCS) and dimethyl sulfoxide (DMSO) were purchased from Gibco, Grand Island, NY, USA; Cytokine interleukin-2 (IL-2), interleukin-6 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), Immunoglobulin A (IgA) Immunoglobulin G (IgG) and Immunoglobulin M (IgM) detecting ELISA kits were purchased from Changfeng Biotechnology Co., Zhejiang, China. All other chemicals and solvents used were of analytical grade.

2.2. Isolation and purification of polysaccharide from the roots of S. miltiorrhiza

The roots of *S. miltiorrhiza* were dried at 50 °C, cut into small pieces and extracted with 95% ethanol for 24 h in a Soxhlet apparatus to remove small lipophilic and pigment molecules. Subsequently, the degreased residues were extracted with twenty volumes of hot distilled water at 100 °C for 2 h each time ($n = 3$), filtered through gauze and concentrated in a rotary evaporator under reduced pressure. The concentrated supernatant was mixed with four volumes of 95% ethanol and kept at 4 °C in refrigerator for 12 h to precipitate the polysaccharides. The precipitated polysaccharides were dissolved in deionized water and then the protein in polysaccharides was removed by extracting three times with the Sevag reagent (1-butanol/chloroform, 1:4, v/v) (Navarini et al., 1999). The aqueous phase was again dialyzed, concentrated and precipitated with ethanol. After centrifugation at 12,000 rpm at 4 °C, the precipitate was washed with anhydrous ethanol, acetone and ether in turn, and then dried to yield the crude polysaccharides (CSMPS).

The crude polysaccharide was dissolved in 0.1 M NaCl solution, filtered through 0.45-μm Millipore filter, and then the filtrate was subjected to DEAE-52 cellulose column (length: 40 cm, internal diameter: 2.6 cm) chromatography eluting with different concentrations of stepwise NaCl solution (0.1–1.0 M). The elution fractions (5 ml) were collected and monitored for carbohydrate content based on the phenol–sulfuric acid method at 492 nm absorbance (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and three fractions (CSMPA, CSMPB and CSMPC) were obtained. CSMPA was further chromatographed on a Sephadex G-100 gel filtration column (length: 100 cm, internal diameter: 2.6 cm), and eluted with 0.1 M NaCl at a flow rate of 0.5 ml/min. A polysaccharide was obtained, which was dialyzed and lyophilized to give a white purified polysaccharide (named SMPA).

2.3. Compositional analysis

The total carbohydrate content of the purified sample was determined according to the phenol–sulfuric acid method using glucose as a standard (Dubois et al., 1956). Uronic acid content was measured by the carbazole–sulfuric acid method using glucuronic acid as standard (Bitter & Muir, 1962). In addition, protein in the polysaccharides was quantified according to the Lowry method using bovine serum albumin (BSA) as the standard (Lowry, Rosebrough, Farr, & Randall, 1951), combined with UV absorption scanning on a UV–vis spectrophotometer (Shimadzu Corporation, Japan) at 200 to 300 nm.

The average molecular weight of SMPA was determined by HPGPC on a Shimadzu HPLC system equipped with a TSK G3000 PWxl column (7 μm × 7.8 mm × 300 mm) and a refractive index RID-10A detector. The sample was dissolved in mobile phase solution and passed through a 0.45 μm filter before being loaded onto the HPLC column. The detailed experiment conditions were as follows: mobile phase: 0.1 M $NaNO_3$; flow rate: 0.9 ml/min; column temperature: 45 °C; injection volume: 20 μl. The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (6100, 16,500, 26,290, 40,000, 84,000, 158,000) (Sun, Tang, Gu, & Li, 2005).

Neutral monosaccharide composition was analyzed according to the following procedure as described by Dai, Zhu, Tang, Wang, and, Chen (2007). Briefly, SMPA (10 mg/ml) was hydrolyzed in 1 ml of TFA (4 mol/l) at 110 °C in a sealed tube for 2 h. After removing TFA with methanol, the residue was dissolved in NaOH and reacted with PMP to convert the monosaccharides into their PMP derivatives. The PMP derivatives of the ten standard sugars (Man, Rib, Rham, GlcUA, GalUA, Glc, Gal, Xyl, Ara, Fuc) and SMPA were analyzed on a DIKMA Inertsil ODS-3 column (4.6 mm × 250 mm) connected to a Shimadzu HPLC system.

2.4. Animals and treatment

Male Wistar rats of 6-week-old were purchased from the Animal Centre of the Fourth Military Medical University (China). All animal procedures were conducted in compliance with the guidelines approved by the China Association of Laboratory Animal Care and the Institutional Animal Care Committee of the Fourth Military Medical University.

After 1 week of acclimatization, rats were randomly divided into three groups ($n = 10$). All the animals were given MNNG at dose of 200 mg/kg body weight in drinking water once daily for 15 weeks, except for the normal group. The day after 15 weeks of oral administration of MNNG was taken as day zero. Group I served as the normal control and were given distilled water orally for 6 weeks. Groups II served as the model control and were given distilled water orally for 6 weeks. Groups III received SMPA (200 mg/kg body weight, dissolved in distilled water) throughout the study period. All these treatments were given after the tumor induction, once daily for 6 weeks. The body weights of the animals from each group were recorded at the beginning and at the termination of the experiments.

After the last dose and 24 h fasting, all rats were killed by cervical dislocation. Blood was collected, and the plasma separated was used for analysis. At the same time, thymus and spleen were taken from rats. The impact on immune organ was evaluated based on the thymus index and spleen index (Liu et al., 2008).

2.5. Splenocyte proliferation assay

Spleens were aseptically removed from sacrificed rats with scissors and forceps in cold phosphate buffered saline (PBS) and gently homogenized with a loose teflon pestle. Splenocytes (5×10^5 cells/well) were cultured in RPMI-1640 medium supplemented with 10% newborn bovine serum (NBS) at 37 °C in a humid atmosphere with 5% CO_2 in the presence of Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml), or RPMI 1640 medium. After treatment for 72 h, 50 μl of MTT solution (2 mg/ml) was added to every well and the plate was incubated for another 4 h. The plate was then centrifuged at 2000 rpm for 10 min and the untransformed MTT was carefully removed by pipetting. A total of 150 μl DMSO was added to each well. The plate was then shaken until crystals were dissolved and the absorbance was evaluated in an ELISA reader (Bio-Rad, USA) at 570 nm after 15 min.

2.6. Serum cytokine detection

The serum levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IgA, IgG and IgM were determined by commercial ELISA kits (R&D Systems Inc., Minneapolis, USA) according to the indication of the manufacturer. The results were expressed as the quantity per ml plasma.

2.7. NK cell cytotoxicity

The NK cell cytotoxicity was determined using the standard ^{51}Cr-release assay as previously described with some modification (Zeytin et al., 2008). Briefly, YAC-1 cells (target cells) were purchased from American Type Culture Collection (Rockville, MD, USA) and were maintained in RPMI-1640 media supplemented with 10% FCS in a 5% CO_2 incubator at 37 °C to measure NK cells (effector cells) activity. These target cells (1×10^6) were labeled with 250 μCi of $Na_2[^{51}Cr]O_4$ for 30 min at 37 °C, washed, and resuspended at a concentration of 1×10^5 cells/ml. Thereafter, the NK cells (100 μl) collected from control or SMPA-treated groups were mixed with $Na_2[^{51}Cr]O_4$ labeled Yac-1 cells (100 μl) at an effector to target (E:T) ratio of 25:1 in 96-well plates. After incubation for 5 h at 37 °C in an atmosphere of 5% CO_2, the cell-free supernatants were harvested and the radioactivity was counted by a Beckman 5500 gamma counter (Beckman Scientific Instruments, Irvine, CA). Specific cellular cytotoxicity was calculated by the following formula: percentage of specific lysis = (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100. Controls for spontaneous or maximal ^{51}Cr release were target cells in medium or in 1.5% Triton, respectively. Spontaneous cpm was the radioactivity from wells with target cells alone. All assays were done in triplicate.

2.8. Cytotoxic T lymphocytes (CTL) assay

HGC-27 cells (target cells) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco modified eagle's medium (DMEM) containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at in a 5% CO_2 humidified incubator at 37 °C. The CTL activity was analyzed using standard ^{51}Cr-release method as described above. Briefly, spleen cells (2×10^6) were co-cultured with 2×10^5 irradiated HGC-27 cells for 5 days to yield CTL (effector cells). After 5 days, CTL were harvested and cocultured with $Na_2[^{51}Cr]O_4$ labeled HGC-27 cells (1×10^4 cells/well) at effector-to-target (E:T) ratio of 25:1 for 5 h in 96-well round-bottom plates. Target cell killing was determined by a Beckman 5500 gamma counter. Percent cytotoxicity was calculated as described above.

2.9. Determination of phagocytotic function of macrophage

After the last dose and 24 h fasting, an individual mouse from each group was intraperitoneally injected with chicken-red cells (1 ml, 5%). After 12 h, the rats were sacrificed and 2 ml normal saline was injected into the abdominal cavity of each mouse. Then the peritoneal fluid (1 ml) kneaded from the abdomen of each mouse was collected and a drop placed on a glass slide. After incubating for 30 min, peritoneal fluid was fixed with a mixture of acetone/methanol (1:1, v/v) and stained with 4% Giemsa for 3 min. After drying, peritoneal macrophages were counted under microscope. The effect of CE-SB on phagocytosis of enterocoelia macrophage was evaluated by the chicken-red cell phagocytic rate and phagocytic index (Liu et al., 2008), which were calculated using the following formulas: phagocytotic rate = (macrophages which phagocyted RBC/total macrophages) × 100%; phagocytotic index = chicken-red cells phagocyted/total macrophages.

2.10. Determination of intracellular granzyme-B and IFN-γ in mouse splenocytes

The splenocytes (1×10^6) obtained from control or SMPA-treated groups were cultured in 5% CO_2 incubator for 12 h. After incubation, cells were washed twice with PBS and re-suspended in cell fixation buffer using 100 μl/1×10^6 cells for 15 min. Again cells were washed once in PBS and resuspended in permeabilization buffer using 50 μl/1×10^6 cells. Anti-mouse granzyme-B-phycoerythrin (PE) antibody was added for incubation for 30 min in dark. After incubation, samples were washed twice with PBS and resuspended in 500 μl of PBS. Twenty-thousand events were analyzed in a Facscalibur flow cytometer using Cell Quest software. The concentrations of the cytokines IFN-γ in supernatants of splenocytes were determined by commercial ELISA kits.

2.11. Statistical analysis

Mean data values are presented with their deviation (mean ± SD). Analysis of variance (ANOVA) was followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $P < 0.05$ for all tests.

3. Results

3.1. Isolation and characterization of SMPA

The crude polysaccharide from the roots of *S. miltiorrhiza* was isolated and purified by DEAE-52 cellulose chromatogram into the three polysaccharides CSMPA, CSMPB and CSMPC (Fig. 1A). The yield of crude polysaccharide CSMPS was 5.23% of the plant raw material, and the yields of three polysaccharides were 41.34%, 9.53% and 10.94% for CSMPA, CSMPB and CSMPC of the parent polysaccharide CSMPS, respectively. Considering its high yield and the need for considerable quantities of material for animal experiments, CSMPA was chosen for further fractionation by a column of Sephadex G-100 gel filtration chromatography, giving a homogeneous polysaccharide SMPA (Fig. 1B) with an average molecular weight of 43 kDa as determined by HPGPC. The total sugar content and uronic acid content of SMPA were 91.67% and 13.14%, respectively. SMPA had one absorption peak at 200 nm and had no absorption at 260 and 280 nm in the UV spectrum, indicating the absence of protein and

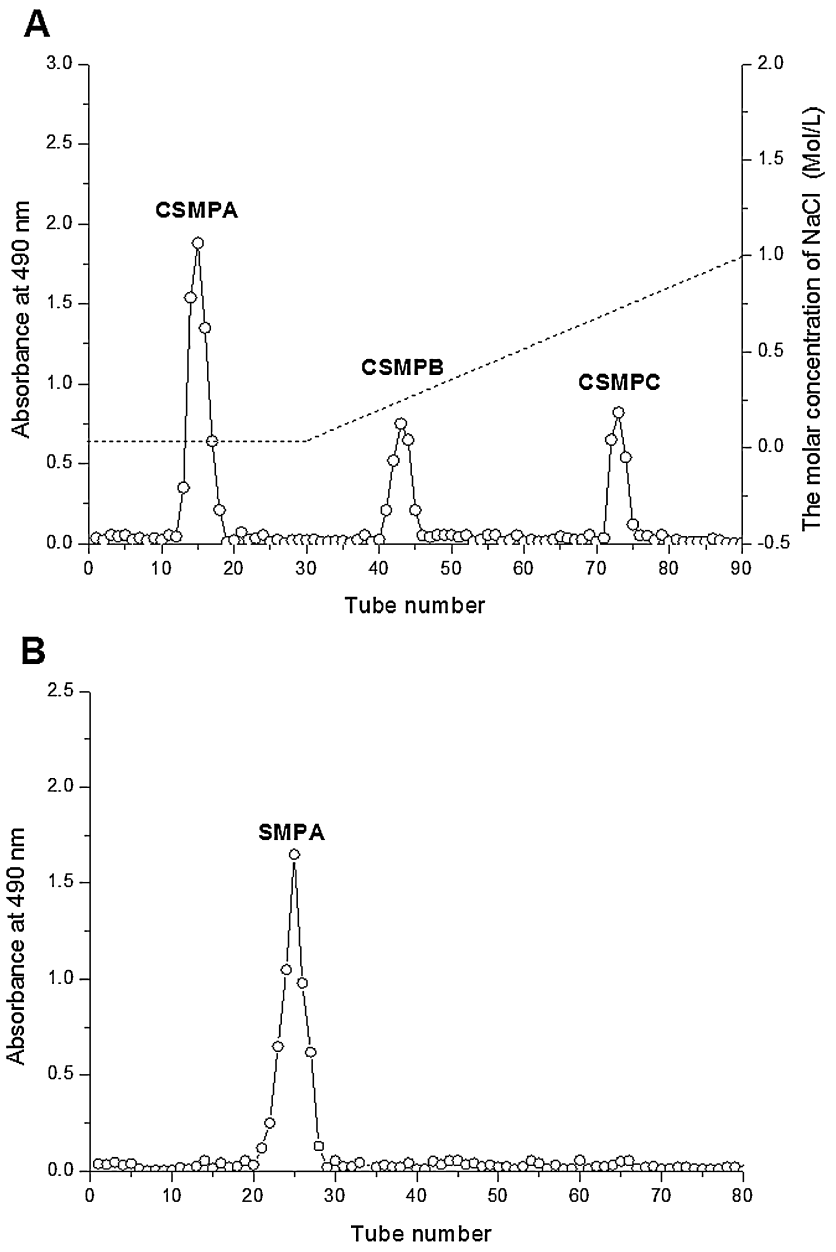

Fig. 1. (A) The profile of three polysaccharides (CSMPA, CSMPB and CSMPC) isolated from the crude polysaccharides (CSMPS) of *S. miltiorrhiza* on a DEAE-52 cellulose column (B) The profile of a homogeneous polysaccharide (SMPA) isolated from CSMPA on a Sephadex G-100 column.

nucleic acid. GC analysis showed that SMPA was composed of Gal, Glc, Rham, Man, and GalUA in a molar ratio of 2.14:1.42:1.16:2.15:1 (Table 1).

3.2. Effect of SMPA on organ indices and body weight in gastric cancer rats

In the normal group, the rats looked perfectly relaxed, moved quickly and ate well. Their hair looked bright, and faces were hard and granular. In the rats of MNNG-treated groups, the autonomic movement gradually became slower, with lusterless hair, loss of appetite and slow weight gain; in contrast, rats in the SMP-treated groups were vigorous with shiny fur and rapid body weight increases, showing a good general condition. Pathological examination showed that atrophy and dysplasia of the gastric mucosa were manifest in MNNG-treated rats, indicating that gastric cancer model had been successfully established (data not shown).

As shown in Table 2, the body weights of rats in different groups didn't differ from each other before experiment ($P > 0.05$). Until the end of the experiment, the body weights of rats in the model group were significantly lower than those in the normal control group ($P < 0.05$). An increase of average body weight was observed in the SMPA-treated rats compared to that of the model control rats ($P < 0.05$). More importantly, SMPA could significantly increase the relative spleen and thymus weight of rats ($P < 0.05$ or $P < 0.01$) compared to the model group in a dose dependent manner. However, the spleen index in MNNG-treated group was lower than that of the normal control ($P < 0.01$), indicating the suppressing effect of tumor on immune system as reported previously. Taken together with these data, the results implied SMPA not only increased the body

Table 1
The physicochemical properties of SMPA.

Sample	Carbohydrate (wt%)[a]	Protein (wt%)[a]	Uronic acid (wt%)[a]	Yield (wt%)[b]	Molecular weight (kDa)	Sugar components (mol.%)[c]					
						Galactose	Glucose	Rhamnose	Manose	Galacturonic acid	
SMPA	91.67	/	13.14	41.34	43	2.14	1.42	1.16	2.15	1	

[a] The contents of carbohydrate, protein and uronic acid were calculated as wt%.
[b] Value is expressed as wt% of the parent crude polysaccharide CSMPS.
[c] The quantities of the sugars was given in mol.%.

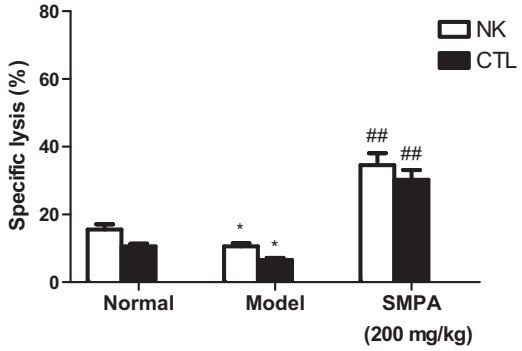

Fig. 2. Effect of SMPA on NK cells and CTL activities in gastric cancer rats. Values are expressed as means ± S.D. ($n = 10$); * $P < 0.05$ as compared with the normal group; ## $P < 0.01$ as compared with the model control.

weight but also improved the immune organ indices in MNNG-treated model rats.

3.3. Effect of SMPA on splenocyte proliferation in gastric cancer rats

The ability of lymphocytes to respond to mitogen reflects the immune potential of the organism (Zeng et al., 2013). To investigate whether SMPA enhanced the immune potential of the organism, we measured the effect of SMPA on mitogen-stimulated splenocyte proliferation in gastric cancer rats. As shown in Table 3, Con A- and LPS-stimulated splenocyte proliferation in the MNNG-treated group were significantly lower than those of the normal control ($P < 0.05$ or $P < 0.01$). However, SMPA showed a significant comitogenic activity on ConA- or LPS-stimulated lymphocytes ($P < 0.01$) at the dose of 200 mg/kg. In the abesence of mitogen (Con A or LPS), SMPA also showed a moderate mitogenic activity for splenocyte proliferation, suggesting that it had direct benefit to splenocyte proliferation. The present results indicated that SMPA had directly mitogenic or comitogenic effect on mouse splenocytes proliferation to strengthen immunological response and improve immunity.

3.4. Effect of SMPA on the serum levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IgA, IgG and IgM in gastric cancer rats

As seen in Table 4, IL-2, IL-4, IL-10, IgA, IgG and IgM levels in MNNG-treated model control rats were significantly lower than those in normal control rats ($P < 0.05$ or $P < 0.01$), whereas cytokine IL-6 and TNF-α levels in MNNG-treated model control rats were markedly higher than those in normal control rats ($P < 0.01$). This indicated that immune function had decreased in gastric cancer model control rats. SMPA treatment had significantly increased blood IL-2, IL-4, IL-10, IgA, IgG and IgM levels and reduced the IL-6 and TNF-α levels in gastric cancer rats as compared to those in gastric cancer rats ($P < 0.05$ or $P < 0.01$). These results suggested that SMPA treatment could improve immune function in gastric cancer rats partly through promoting IL-2, IL-4, IL-10, IgA, IgG and IgM production and inhibiting cytokine IL-6 and TNF-α secretion.

3.5. Effect of SMPA on NK cell cytotoxicity and CTL response in gastric cancer rats

Tumor cell elimination is known to be mediated in part by the cytotoxic activity of NK cells and CTL (Sunila, Hamsa, & Kuttan, 2011). Therefore, we measured the effects of SMPA on the cytotoxic activities of NK cells and CTL, and the results were shown in Fig. 2. Clearly the activities of NK cells and CTL in gastric cancer model control rats were suppressed and significantly lower than those of

Table 2
Effect of SMPA on body weight and the immune organ indices in gastric cancer rats.

Group	Dosage (mg/kg/d)	Body weight (g)		Increase of body weight (g)	Spleen index (mg/g)	Thymus index (mg/g)
		Pre-treatment	Post-treatment			
Normal control	/	186.2 ± 13.8	228.5 ± 20.1	42.3	7.55 ± 0.68	3.01
Model control	/	186.4 ± 15.9	201.8 ± 17.3	15.4[a]	4.39 ± 0.34[aa]	3.08
SMPA group	200	186.1 ± 18.1	225.1 ± 18.9	39.0[b]	8.48 ± 0.87[bb]	3.56[b]

Values are expressed as means ± S.D. (n = 10).
[a] P < 0.05.
[aa] P < 0.01 as compared with the normal group.
[b] P < 0.05.
[bb] P < 0.01 as compared with the model control.

Table 3
The effect of SMPA on lymphocyte proliferation in gastric cancer rats.

Group	Dosage (mg/kg/d)	Lymphocytes		
		Medium	ConA	LPS
Normal control	/	0.34 ± 0.03	0.40 ± 0.03	0.43 ± 0.03
Model control	/	0.28 ± 0.02[a]	0.29 ± 0.03[aa]	0.30 ± 0.03[aa]
SMPA group	200	0.43 ± 0.04[bb]	0.56 ± 0.05[bb]	0.59 ± 0.04[bb]

Values are expressed as means ± S.D. (n = 10).
[a] P < 0.05.
[aa] P < 0.01 as compared with the normal group.
[bb] P < 0.01 as compared with the model control.

the normal control (P < 0.05), while a significant increase of activity was observed in SMPA-treated groups at the dose of 200 mg/kg as compared to those in gastric cancer rats (P < 0.01). These results further revealed that SMPA could strengthen immune function by evoking NK cells and CTL activation in gastric cancer rats.

3.6. *Effect of SMPA on phagocytotic function of macrophages in gastric cancer rats*

Macrophages are considered to be among the pivotal immunocytes of the host defense against tumor growth (Fidler & Kleinerman, 1993). The phagocytotic function of macrophages can reflect the immune function to some extent (Wu et al., 2004). As shown in Table 5, compared with the normal control group, MNNG treatment decreased the phagocytic index and phagocytic rate significantly (P < 0.05). However, the phagocytotic function of macrophages was significantly increased in the 200 mg/kg group compared to the MNNG-treated model control (P < 0.05 or P < 0.01). SMPA oral administration at a dose of 200 mg/kg increased the phagocytotic rate and the phagocytic index to 45.04% and 0.87, respectively. The results suggested that SMPA treatment contributed to the great improvement or enhancement of the

Table 4
Effect of SMPA on the serum levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IgA, IgG and IgM in gastric cancer rats.

	Normal control	Model control	SMPA group (200 mg/kg)
IL-2 (ng/ml)	6.57 ± 0.68	3.12 ± 0.51[aa]	6.14 ± 1.31[bb]
IL-4 (ng/ml)	10.25 ± 1.20	6.52 ± 0.87[aa]	9.87 ± 1.51[bb]
IL-6 (ng/ml)	56.73 ± 5.21	69.56 ± 4.32[aa]	59.56 ± 5.43[b]
IL-10 (ng/ml)	52.87 ± 5.04	36.82 ± 6.58[a]	51.52 ± 6.84[b]
TNF-α (ng/ml)	7.10 ± 0.72	14.24 ± 0.46[aa]	7.82 ± 0.58[bb]
IgA (g/l)	0.68 ± 0.06	0.41 ± 0.03[a]	0.63 ± 0.05[b]
IgG (g/l)	6.35 ± 0.54	4.25 ± 0.38[a]	6.01 ± 0.51[b]
IgM (g/l)	0.74 ± 0.06	0.46 ± 0.03[a]	0.75 ± 0.06[b]

Values are expressed as means ± S.D. (n = 10).
[a] P < 0.05.
[aa] P < 0.01 as compared with the normal group.
[b] P < 0.05.
[bb] P < 0.01 as compared with the model control.

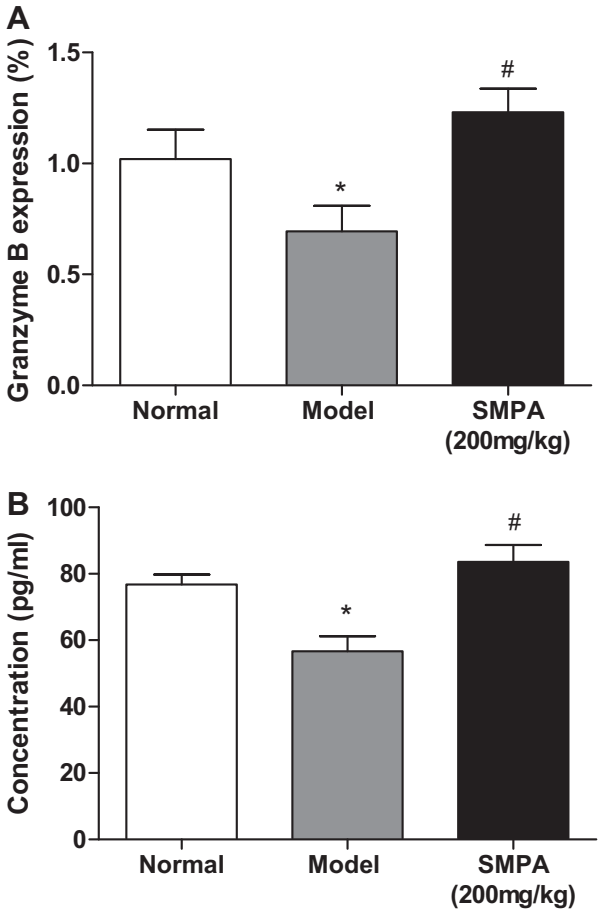

Fig. 3. (A) Effect of SMPA on the expression of granzyme-B in rat splenocytes; (B) effect of SMPA on the production of IFN-γ in rat splenocytes. Values are expressed as means ± S.D. (n = 10); * P < 0.05 as compared with the normal group; # P < 0.05 as compared with the model control.

Table 5
Effect of SMPA on phagocytic function of intra-abdominal macrophages in gastric cancer rats.

Group	Dosage (mg/kg/d)	Phagocytic rate (%)	Phagocytic index
Normal control	/	35.01 ± 3.24	0.66 ± 0.05
Model control	/	20.23 ± 2.54[a]	0.41 ± 0.04[a]
SMPA group	200	45.04 ± 4.70[bb]	0.87 ± 0.06[b]

Values are expressed as means ± S.D. ($n = 10$).
[a] $P < 0.05$ as compared with the normal group.
[b] $P < 0.05$.
[bb] $P < 0.01$ as compared with the model control.

phagocytic function of macrophages in gastric cancer rats, suggesting that SMPA could improve the quality of life of patients suffering with malignant disease under clinical conditions.

3.7. Effect of SMPA on intracellular granzyme-B and IFN-γ production in rat splenocytes

Significantly lower production of granzyme-B and IFN-γ ($P < 0.05$) was found in the splenocyte cultures of gastric cancer rats than those in normal rats. However, treatment with SMPA at 200 mg/kg induced a significant increase in expression of granzyme-B (Fig. 3A) or IFN-γ (Fig. 3B), showing that SMPA activates total intracellular granzyme-B and IFN-γ content which helps in killing of the infected target cells or cancerous cells.

4. Discussion and conclusions

The immune system plays an important role in antitumor defense (Zamarron & Chen, 2011; Zhang et al., 2011). Many studies have suggested that the antitumor activity of the polysaccharides from several traditional Chinese herbs might be achieved by improving immunity (Gao, Bi, Ma, & Lu, 2010; Jeong, Koyyalamudi, Jeong, Song, & Pang, 2012; Wenner et al., 2012). The discovery and identification of new antitumor drugs, which can potentiate the immune function has become an important goal of research in immunopharmacology and oncotherapy (Yuan, Song, Li, Li, & Dai, 2006). Therefore, we isolated the purified polysaccharide SMPA from the roots of *S. miltiorrhiza* and investigated the effect of SMPA on the immune response in gastric cancer rats to analyze the underlying mechanism of its antitumor activity. MNNG was used successfully to induce gastric cancer in rats by intragastric administration for 15 continuous weeks. Administration of SMPA (200 mg/kg) for another 6 weeks not only increased the body weight, but also improved the immune organ indices, such as spleen and thymus. These results indicated that SMPA had an antitumor effect via activation of the immune response of the host organism and showed no evident side effects.

Lymphocyte proliferation is the most direct indicator of the state of host immunity (Tu, Sun, & Ye, 2008). The body's immune response to a tumor includes both the cellular and humoral immunity (Reuschenbach, von Knebel Doeberitz, & Wentzensen, 2009). The humoral immune response by B lymphocyte is a specific antigen antibody reaction. Cell-mediated immune defense was mediated specifically by T lymphocyte, which plays an important role in enhancing the immune function of the organism (Ali, Kumar, Naqvi, & Rao, 2013). The capacity to elicit effective T- and B-cell immunity can be shown by the stimulation of the lymphocyte proliferation response (Marciani et al., 2000). It is generally known that Con A stimulates T-cells and LPS stimulates B cell proliferation. The proliferation assay showed that SMPA could significantly promote the Con A-, LPS or medium-stimulated splenocyte proliferation in gastric cancer rats. These findings indicated that SMPA contributed to the activation of T- and B-cells and enhanced the humoral and cell-mediated immunity in gastric cancer rats.

Cytokines regulate both cellular and humoral immune responses by affecting immune cell proliferation, differentiation and functions (Belardelli & Ferrantini, 2002). Cytokines such as interferons, interleukin and tumor necrosis factor have been widely tested in the treatment of cancer (Lippitz, 2013). Pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and GMCSF act as autocrine growth factors for tumor angiogenesis. These cytokines could be pro-metastatic or pro-angiogenic and upregulation of these cytokines was closely linked to chronic inflammation and cancer (Aggarwal, Shishodia, Sandur, Pandey, & Sethi, 2006; Obameso, Akinyele, Oladunmoye, & Osho, 2011; Polat, 2011). Along with the pro-inflammatory cytokines, there are also anti-inflammatory cytokines (IL-2, IL-4, IL-10). These have the opposite effect, in that they help to limit any inflammation present. IL-2 is an important cytokine excreted by the activated T cells, which exhibit high cytolytic activities against autologous tumor cells (Xu et al., 2009a). IL-4 has been shown to have a modest but direct inhibitory effect on the growth of various tumor cells in vitro and in vivo, including human melanoma, non-Hodgkin's malignant B-lymphoma, and colon, renal, gastric, and breast carcinoma (Luo & Wu, 2011). IL-10 is an important immunoregulatory cytokine that inhibits T cell function by suppressing the expression of proinflammatory cytokines (mostly Th1 cytokines), such as IL-1β, IL-6, TNF-α, IL-8, and IL-12 (de Waal Malefyt et al., 1991). Furthermore, three major immunoglobulin classes (IgG, IgA and IgM) have been demonstrated to provide the bulk of immunity to most infectious agents or tumors (Staff et al., 2012). Our present results showed that SMPA treatment could increase blood IL-2, IL-4, IL-10 levels and reduced the IL-6 and TNF-α levels in gastric cancer rats. In addition, SMPA treatment could still enhance blood IgA, IgG and IgM levels in gastric cancer rats. These results suggested that SMPA treatment could improve immune function in gastric cancer rats partly through inhibiting pro-inflammatory cytokine production and promoting anti-inflammatory cytokine and immunoglobulin antibody production.

NK cells and CTL represent two major populations of cytotoxic lymphocytes (Xu, Yao, Sun, & Wu, 2009b), and play a major role in the defense against both tumors and viruses (Andersen, Schrama, Thor Straten, & Becker, 2006; Boon et al., 1994; Kawai & Akira, 2006; Smyth et al., 2005). They are both able to kill autologous cells infected with intracellular pathogens, as well as tumor cells. NK cells are a type of cytotoxic lymphocyte critical to the innate immune system. The role NK cells plays is analogous to that of cytotoxic T cells (CTL) in the vertebrate adaptive immune response (Vivier et al., 2011). Most CTL expressing T-cell receptors (TCRs) can recognize a specific antigen often produced by cancer cells or viruses. Unlike CTL, however, the killing by NK cells is non-specific. NK cells do not need to recognize antigen/MHC on the target cells and can react against and destroy a target cell without prior sensitization to it. We therefore measured the cytotoxic activities of NK and CTL using ^{51}Cr release assay. Our present results found that SMPA caused an enhancement of the killing activity of NK cells and CTL from splenocytes in gastric cancer rats, suggesting SMPA could enhance the specific and non-specific cytolytic activities against tumorigenesis in vivo.

Macrophage is the most important phagocyte, and plays an essential and pivotal role in host defense against any type of invading cells including tumor cells (Katsiari, Liossis, & Sfikakis, 2010). Macrophages protect the host by phagocytosis, present antigens to lymphocytes and to release numerous cell factors that regulate the activity of other cells (Jiao et al., 2009). In the experiment, the polysaccharide SMPA significantly enhanced the phagocytotic functions of peritoneal macrophages in gastric cancer rats, which

implied that this immune response may also contribute to the anti-tumor mechanism of SMPA in vivo.

Furthermore, SMPA markedly increased intracellular levels of granzyme-B in rat splenocytes, indicating that SMPA increased NK/CTL activities at least partially via increase in the intracellular level of this cytolytic molecule, showing its anticancer property. IFN-γ is the hallmark cytokine of Th1-type $CD4^+$ T cells with anti-tumor and immunomodulatory properties (Blankenstein & Qin, 2003). IFN-γ could inhibit cell proliferation and angiogenesis in the tumor microenvironment (Masuda et al., 2009; Wang, Zhu, & Lin, 2012). In the present study, the content of IFN-γ secreted by splenocytes in gastric cancer rat was significantly increased by SMPA. The increase may also explain the antitumor properties of this polysaccharide.

At this stage of our study, our results provide a clue that SMPA appears to be worthy of consideration as an efficacious adjacent immunopotentiating agent in the treatment of tumor where immunosuppression occurs. Further investigation is necessary to elucidate and confirm the effectiveness of restoring the immune response by SMPA in the treatment of tumors.

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