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Journal of Cellular Biochemistry 112:860–871 (2011)

Journal of Cellular Biochemistry

Ganoderma atrum Polysaccharide Induces Anti-Tumor Activity Via the Mitochondrial Apoptotic Pathway Related to Activation of Host Immune Response

Wen-Juan Li,[1] Yi Chen,[1] Shao-Ping Nie,[1] Ming-Yong Xie,[1*] Ming He,[1,2] Shen-Shen Zhang,[1] and Ke-Xue Zhu[1]

[1] *State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China*

[2] *Department of Pharmaceutical Science, Nanchang University, Nanchang 330006, China*

ABSTRACT

Ganoderma atrum polysaccharide (PSG-1), the major active ingredient isolated from *Ganoderma atrum*, has been suggested as a candidate for cancer therapy. The aim of this study was to investigate the anti-tumor effect of PSG-1 using sarcoma 180 (S-180) transplanted mice and further to examine the molecular mechanisms of PSG-1-induced anti-tumor effect. Results showed that PSG-1 significantly inhibited tumor growth in S-180-bearing mice. PSG-1-induced tumor apoptosis was associated with the alteration of Bcl-2 family proteins, increase of reactive oxygen species generation, loss of mitochondrial membrane potential ($\Delta\psi_m$), release of cytochrome *c* from the mitochondria into cytosol, and activation of caspase-3 and -9. Elevation of immune function was also shown during PSG-1-induced tumor apoptosis, as evidenced by increase of spleen and thymus indexes, lymphocyte proliferation, concentrations of tumor necrosis factor (TNF)-α, and interleukin-2 in serum. Furthermore, the combined treatment of PSG-1 and cyclophosphamide (CTX) results in an enhancement of the anti-tumor effect of CTX alone via increased host immune response. These results suggested that PSG-1 had a potent anti-tumor activity by induction of tumor apoptosis through mitochondrial pathways, and immunoenhancement effect of PSG-1 was related to its anti-tumor effect. In addition, PSG-1 enhanced CTX-induced anti-tumor activity in S-180-bearing mice. J. Cell. Biochem. 112: 860–871, 2011.

KEY WORDS: GANODERMA ATRUM POLYSACCHARIDE; ANTI-TUMOR; APOPTOSIS; MITOCHONDRIA; IMMUNNOMODULATION

Cancer is becoming a leading killer for humans. Surgery, radiation, and chemotherapy are the mainstay of cancer treatments [Sarkar and Li, 2006]. However, severe adverse effects such as serious infection due to anticancer drugs are also major problems in the clinical setting. In particular, side effects of drugs might be fatal in immunocompromised patients. Immune-based approaches that recruit the host anti-tumor immune response to the therapeutic effort are particularly attractive strategies for improving clinical outcomes in malignant disease [Eisenbrand, 2006; Vujanovic and Butterfield, 2007]. Therefore, drugs enhancing anti-tumor activities and the condition of the immune system in tumor-bearing organisms are of great interest. Screening for natural products with specific health benefits for immunological activities has been a fast growing sector in cancer research [Fidler and Ellis, 2006; Xu et al., 2009].

Ganoderma atrum belongs to the polyporaceae family of Basidiomycota. This fungus has been widely used in Asian countries for centuries to prevent or treat a variety of diseases, especially cancer. In the recent 30 years, many studies have demonstrated the anti-tumor effect of *G. atrum* and attributed it to the activated host immune response. Its polysaccharides have been regarded as the major bioactive substances and responsible for the anti-tumor effect of *Ganderma atrum* [Gao et al., 2004, 2005; Lin et al., 2006; Paterson, 2006], but there is little investigation about the anti-tumor effect of its polysaccharides and the mechanisms in this pathological condition.

Sarcoma 180 (S-180) bearing mice have been one of the classical solid tumor models for a very long time. Naturally occurring polysaccharides have been reported to exert anti-tumor activity in S-180 bearing mice [Yuan et al., 2006]. However, molecular

Grant sponsor: National High Technology Research and Development Program of China (863 Program); Grant number: 2008AA10Z325; Grant sponsor: Objective-Oriented Project of State Key Laboratory of Food Science and Technology; Grant number: SKLF-MB-200806.

*Correspondence to: Prof. Ming-Yong Xie, State Key Laboratory of Food Science and Technology, Nanchang University, 235 Nanjing East Road, Nanchang 330047, China. E-mail: myxie@ncu.edu.cn

Received 28 April 2010; Accepted 30 November 2010 • DOI 10.1002/jcb.22993 • © 2010 Wiley-Liss, Inc.
Published online 7 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

mechanisms for the anti-tumor effect of polysaccharide are far from clear. Apoptosis, or programmed cell death, is a physiological process of cell death, which has been identified to be an important molecular basis for both the initiation and progression of cancer [Burger, 2004; Stein, 2005; Jiang et al., 2006; Mohapatra et al., 2009]. Two major pathways that are associated with apoptosis have been reported: (a) mitochondria-dependent pathway (intrinsic pathway) and (b) death receptor-dependent pathway (extrinsic pathway) [Eichele et al., 2006; Nicolson, 2007]. It is well-established that the mitochondria-dependent apoptotic pathway is stimulated in anti-tumor treatment both in vitro and in vivo. Mitochondrial damage is associated with the release of cytochrome *c* and activation of caspase-9. Activated caspase-9 leads to the activation of caspase-3, which executes apoptosis [Chen and Chang, 2009; Dasgupta et al., 2009].

A polysaccharide, named PSG-1 with a purity of >99.8%, has been recently isolated from *G. atrum* in our laboratory. Its primary structural features and molecular weight were characterized [Chen et al., 2008]. Therefore, the present study was designed to evaluate the anti-tumor effect of PSG-1 in vivo using S-180-bearing mice and further examined the molecular mechanisms about the effect of PSG-1.

MATERIALS AND METHODS

REAGENTS

PSG-1 was extracted and purified by our laboratory following a published method [Chen et al., 2008]; Cyclophosphamide (CTX) was purchased from Hualian Pharmaceutical Co. Ltd. (Shanghai, China). Cell culture products were obtained from Life Technologies (Paisley, Scotland); 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and Rhodamine-123 (Rho-123) were purchased from Molecular Probes Inc. (OR); Annexin V-FITC apoptosis detection kits were obtained from Becton Dickinson Biosciences (SD); The antibodies against Bcl-2, Bax, cytochrome *c*, and β-actin, as well as the HRP-labeled secondary antibody, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-voltage-dependent anion channel (VDAC) 1 was purchased from Abcam (Cambridge, MA).

CELLS AND ANIMALS

Murine S-180 cell line was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. These cells were cultured in RPMI-1640 medium with 2.0 g/L sodium bicarbonate plus 10% fetal bovine serum, 1×10^5 units/L penicillin, and 100 mg/L streptomycin, and maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C. Kunming mice, weighed 20.0 ± 2.0 g (grade II, Certificate Number SCXK (gan) 2006–0001) were purchased from Jiangxi College of Traditional Chinese Medicine, Jiangxi, China. All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85-23, revised 1996), and all procedures were approved by the Nanchang University Medical College Animal Care Review Committee.

ESTABLISHMENT OF S-180 MODEL

The S-180 model used in the present study was in accordance with a method reported previously [Pal et al., 2009]. Briefly, under sterile conditions, the S-180 mouse sarcoma cells with ascites were harvested, and diluted with sterilized saline. A 0.2 ml of S-180 cell suspension (approximately 1×10^7 cells/ml) was subcutaneously inoculated into mice in the axillary region.

EXPERIMENTAL GROUPS AND ASSAY OF ANTI-TUMOR ACTIVITY IN S-180-BEARING MICE

After 1 week of acclimatization, intact mice were randomized into five experimental groups (n = 8 per group). The experimental groups were divided as follows: (i) Control group: after the day of inoculation (day 1), the mice were injected intraperitoneally (i.p.) once daily with the same volume of 0.9% sodium chloride for 10 consecutive days. (ii) PSG-1-25 group: after the day of inoculation (day 1), PSG-1 (25 mg/kg body weight) was injected i.p. once daily for 10 days in a volume of 0.2 ml. (iii) PSG-1-50 group: after the day of inoculation (day 1), PSG-1 (50 mg/kg body weight) was injected i.p. once daily with the same volume for 10 consecutive days. (iv) PSG-1-100 group: After the day of inoculation (day 1), PSG-1 (100 mg/kg body weight) was injected i.p. once daily with the same volume for 10 consecutive days. (v) Normal group: the mice without inoculating received the same volume of sodium chloride 0.9% injection. All the mice were sacrificed on day 11. The excised tumors were weighted, and the inhibition rate (TIR) was calculated by the following equation:

$$\mathrm{TIR}\,(\%) = \frac{W_C - W_T}{W_C} \times 100$$

where W_C is the average tumor weight in the S-180-bearing control group and W_T is the average tumor weight in the drug-treated groups.

PREPARATION OF MURINE S-180 CELLS IN THE TUMOR OF S-180-BEARING MICE

The tumor was harvested in a germ-free condition and placed in calcium- and bicarbonate-free Hanks' buffer with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The tumor tissues were minced. Single-cell tumor suspensions were pooled in a serum-free RPMI-1640 medium by filtering the suspension through sieve mesh with the aid of a glass homogenizer to exert gentle pressure on tissue fragments. Samples were washed twice in phosphate buffered saline (PBS). After centrifugation (200*g*, 5 min), the cells were resuspended to a concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum.

ANTIPROLIFERATIVE EFFECT OF PSG-1 IN THE TUMOR OF S-180-BEARING MICE

Antiproliferative effect was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide (MTT) assay. The S-180 cells in the tumor of S-180-bearing mice were harvested and seeded in 96-well plates at 5×10^4/well (100 μl/well). The S-180 cells in each well were treated with 10 μl MTT dye (5 mg/ml). After additional 4 h incubation, a total of 200 μl DMSO was added to each well and shaken until the crystal were dissolved [Xu et al., 2008]. The purple formazan crystals were detected at 570 nm using a microplate reader (Bio-Rad Laboratories, CA) and the cell viability was expressed as a percentage of the control.

FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS IN THE TUMOR OF S-180-BEARING MICE

Apoptosis was determined by Annexin V and propidium iodides (PI) double staining. The cells were washed twice and adjusted to a concentration of 1×10^6 cells/ml with ice cold PBS, and stained with Annexin V-FITC and PI in binding buffer (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$). Ten thousand events were collected for each sample. Stained cells were analyzed using a FACStar Plus flow cytometry (Becton Dickinson).

DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi_M$) IN THE TUMOR OF S-180-BEARING MICE

Changes in $\Delta\psi_m$ were assayed by Rho-123 (Molecular Probe) staining in accordance with a procedure described by Li et al. [2010]. Rho-123 fluorescence was measured using a FACStar Plus cell sorter with excitation and emission wavelengths of 488 and 530 nm, respectively. In total, >10,000 events were analyzed. Additionally, $\Delta\psi_m$ depletion was confirmed by another dye, 5,50′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Beyotime Institute of Biotechnology, Shanghai, China). The $\Delta\psi_m$ is detected according to the changes of JC-1 fluorescence. The values of optical density (OD) at 590 and 530 nm were determined by a spectrofluorometry. As the $\Delta\psi_m$ is proportional to the ratio of OD590 to OD530, the $\Delta\psi_m$ was expressed as OD590/OD530 [Huang et al., 2010].

DETERMINATION OF Bcl-2, BAX AND CYTOCHROME *C* PROTEINS EXPRESSION IN THE TUMOR OF S-180-BEARING MICE

Bcl-2, Bax, and cytochrome *c* proteins in tumor were assessed by Western blotting. Briefly, the tumor was fractionated into mitochondrial and cytoplasmic compartments with a mitochondrial/cytosol fractionation kit (Biovision, CA). Samples were mixed with sample buffer (Laemmli sample buffer; Bio-Rad) and then boiled for 5 min and loaded onto 12% or 15% SDS–polyacrylamide gels. After electroblotted onto PVDF membranes, the sample blots were blocked for 2 h with 5% non-fat milk powder in TBST solution (25 mM Tris–HCl [pH 7.6], 0.2 M NaCl, and 0.1% v/v Tween 20) at room temperature. After blocking with 5% non-fat milk, the membranes were incubated with a rabbit polyclonal anti-Bcl-2 (1:200 dilution), rabbit polyclonal anti-Bax (1:300 dilution), and mouse monoclonal anti-cytochrome *c* (1:300 dilution) antibody, respectively. To assure equivalent protein loading, the membranes were also incubated with anti-α-tubulin mouse monoclonal antibody (1:500 dilution), and anti-VDAC1 rabbit polyclonal antibody (1:500 dilution), and subsequently with a corresponding horseradish peroxidase-conjugated second antibody IgG (1:5,000 dilution). The densitometric analysis of bands was carried out using GDS-8000 UVP photo scanner (Upland, CA) and LAB WORK45 Image software.

MEASUREMENT OF CASPASE ACTIVITIES IN THE TUMOR OF S-180-BEARING MICE

Caspase activitives were evaluated by the use of caspase-3 and -9 colorimetric assay kit (Biovision). The assay is based on spectrophotometric detection of the chromophore ρ-nitroanilide (ρNA) after cleavage from the labeled substrate DEVD-ρNA. The absorbance at 405 nm of the released ρNA was monitored in a spectrophotometer.

DETERMINATION OF CLEAVED CAPASE-3 AND -9 PROTEINS EXPRESSION IN THE TUMOR OF S-180-BEARING MICE

The tumor tissues were lysed with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% (v/v) TritonX-100, 1 mM NaF, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Equivalent amounts of protein were resolved on 10% SDS–PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk, the membranes were blotted with anti-cleaved capase-3 rabbit polyclonal antibody (1:1,000 dilution), anti-cleaved capase-9 rabbit polyclonal antibody (1:1,000 dilution; Cell Signaling Technology, Beverly, MA), and detected with a horseradish peroxidase-linked secondary antibody (1:5,000 dilution). The specific bands were detected using chemiluminescence reagents on a chemiluminescence film. Densitometric analysis of the Western blot was performed with GDS-8000 UVP photo scanner and LAB WOEK45 Image software (Bio-Rad). Normalization of results was ensured by running parallel Western blots with anti-β-actin antibody.

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) GENERATION IN THE TUMOR OF S-180-BEARING MICE

The determination of intracellular ROS was based on the oxidation of DCFH-DA to the fluorescent product, 2′,7′-dichlorofluorescein (DCF). The cells were harvested and washed with cold PBS. Washed cells were further incubated with10 μM of DCFH-DA at 37°C for 20 min. Then, DCF fluorescence was monitored by flow cytometry with FACSort cell sorter (Becton Dickinson) at wavelengths of 480 ± 30 nm (excitation) and 535 ± 40 nm (emission).

DETERMINATION OF THYMUS AND SPLEEN INDEXES

After 10 days of administration, mice were sacrificed by cervical dislocation. Spleen and thymus weights of the mice were measured. Thymus and spleen indexes were expressed as the thymus (or spleen) weight (mg) over the body weight (g).

DETERMINATION OF TUMOR NECROSIS FACTOR-(TNF)-α AND INTERLEUKIN (IL)-2 BY ELISA

The concentrations of IL-2 and TNF-α in serum were determined using ELISA kits (SenXiong Biotech, Shanghai, China). All samples and provided standards were analyzed in duplicate. A standard curve was constructed using standards provided in the kits, and the cytokine concentrations were determined from the standard curves using linear regression analysis.

PREPARATION OF LYMPHOCYTE CELLS

The extirpated spleens were treated in germ-free condition. Single-cell spleen suspensions were pooled in serum-free RPMI-1640 medium by filtering the suspension through sieve mesh with the aid of a glass homogenizer to exert gentle pressure on the spleen fragments. Samples were washed twice in PBS/0.1% bovine serum albumin. After centrifugation (200*g*, 5 min), the cells were resuspended to a concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum.

ASSAY OF SPLENOCYTE PROLIFERATION INDUCED BY T- AND B-CELL MITOGENS CONCANAVALIN A AND LIPOPOLYSACCHARIDE, RESPECTIVELY

Splenocytes were placed into the 96-well flat-bottomed microplates in triplicate at 5×10^4 cells/well, then 2.5 μg/well concanavalin A (Con A) or 10 μg/well lipopolysaccharide (LPS) was added to the wells. After incubation for 48 h, 20 μl MTT solution (5 mg/ml) was added to each well. After additional 4 h incubation, the cells were lysed and the purple formazan crystals were solubilized for detection at 570 nm. The absorbance (A) was translated into lymphocyte proliferation ratio for comparison: lymphocyte proliferation ratio = $\text{test}_A/\text{normal control}_A \times 100\%$.

STATISTICAL ANALYSES

Values are expressed as means ± SEM One-way analysis of variance followed by the Student–Newman–Keuls test was applied to determine the statistical significance between various groups. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

EFFECTS OF PSG-1 ON THYMUS, SPLEEN INDEXES AND TUMOR WEIGHTS IN S-180-BEARING MICE

As shown in Figure 1A,B, PSG-1 could significantly inhibit the growth of mouse transplanted S-180. The groups treated with PSG-1 showed an increase of tumor inhibition rate compared to the control group. These results showed that PSG-1 had a potent anti-tumor effect. All the PSG-1 treated groups caused a significant increase in the thymus and spleen indexes compared with control group (Table I).

EFFECTS OF PSG-1 ON THE PROLIFERATION OF TUMOR IN S-180-BEARING MICE

In our present study, we examined whether PSG-1 inhibited the cellular proliferation of tumor in S-180-bearing mice by MTT assay. Compared to control group, PSG-1 significantly inhibited the proliferation of tumor (Fig. 1E). These results indicated that PSG-1 can exert antiproliferative activity in the tumor of S-180-bearing mice.

APOPTOSIS IN THE TUMOR OF S-180-BEARING MICE OWING TO PSG-1 ADMINISTRATION

We thought that the anti-tumor effect of PSG-1 might be mediated through the initiation of apoptosis. Accordingly, the effect of PSG-1 on tumor apoptosis was further investigated by flow cytometric analysis. Flow cytometric analysis using Annexin V and PI double-staining, discriminates intact cells ($\text{AnnexinV}^-/\text{PI}^-$), apoptotic cells ($\text{AnnexinV}^+/\text{PI}^-$), and necrotic cells ($\text{AnnexinV}^+/\text{PI}^+$). Compared to control group, both necrotic cells and apoptotic cells were significantly increased in the tumor administered with PSG-1 (Fig. 1C,D). Therefore, PSG-1-induced anti-tumor effect was related to apoptosis and necrosis in the tumor of S-180-bearing mice.

EFFECTS OF PSG-1 ON $\Delta\Psi_M$ AND THE RELEASE OF CYTOCHROME *C* INTO THE CYTOSOL IN THE TUMOR OF S-180-BEARING MICE

Recent studies have suggested that mitochondria play an essential role in death signal transduction. Mitochondrial dysfunctions induce the release of several apoptogenic proteins, which subsequently causes apoptosis. The loss of $\Delta\psi_m$ is associated with mitochondrial dysfunctions [Kluck et al., 1997]. The $\Delta\psi_m$ was detected with Rho123 staining by flow cytometric analysis. As shown in Figure 2A,B, PSG-1 caused a marked loss of Rho123 fluorescence. Moreover, PSG-1 caused the loss of $\Delta\psi_m$ was confirmed by JC-1 staining (Fig. 2C). JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Mitochondria depolarization is indicated by a decrease in the red to green fluorescence intensity ratio. The $\Delta\psi_m$ was expressed as the ratio of OD590/OD530 in accordance with literature. This was in accordance with the result of Rho123 staining (Fig. 2C). Taken together, these results suggest that PSG-1 is capable of inducing mitochondrial dysfunction.

Mitochondrial dysfunction releases several apoptogenic proteins, most notably cytochrome *c*, from the mitochondria into the cytosol. Western blot revealed that PSG-1 led to the release of cytochrome *c* from the mitochondria into the cytosol (Fig. 3A). These results indicate that PSG-1-induced apoptosis is associated with the mitochondrial dysfunction and the release of cytochrome *c* in the tumor of S-180-bearing mice.

EFFECTS OF PSG-1 ON CASPASE ACTIVITY IN THE TUMOR OF S-180-BEARING MICE

Caspases are a family of cysteine proteases that are synthesized as inactive zymogens and are proteolytically cleaved into subunits during apoptosis [Lin et al., 2009]. We therefore elected to investigate the ability of PSG-1 to modulate caspase-3 and -9 activity. As shown in Figure 4A, Western blot analysis revealed that PSG-1 markedly promoted both caspase-3 (17/19 kDa) and -9 (37 kDa) activation [Lin et al., 2009]. In addition, we next examined the caspase-9 and -3 activities by using caspase-3 and -9 colorimetric assay kit. Both caspase-3 and -9 activities increased after administration of PSG-1 in the S-180-bearing mice (Fig. 4B,C). These results suggested that PSG-1-induced apoptosis is associated with the activation of caspase-3 and -9 in the tumor of S-180-bearing mice.

EXPRESSION OF APOPTOSIS-ASSOCIATED PROTEINS IN THE TUMOR OF S-180-BEARING MICE

To investigate the molecular mechanism of PSG-1-induced apoptosis, the expression of several apoptosis-associated proteins was examined. The expression of Bcl-2 was decreased by PSG-1 treatment (Fig. 3B). Western blot analysis also revealed that mitochondrial Bax protein increased after the administration of PSG-1, together with a decrease in cytosolic Bax (Fig. 3B). These data indicate that PSG-1 induced the loss of Bcl-2 from mitochondria and Bax translocation in the tumor of S-180-bearing mice.

EFFECTS OF PSG-1 ON ROS PRODUCTION IN THE TUMOR OF S-180-BEARING MICE

Mitochondrial production of ROS also seems to play a role in apoptosis [Li et al., 2010]. ROS, the byproducts of mitochondria, has been suggested to regulate the process involved in the initiation

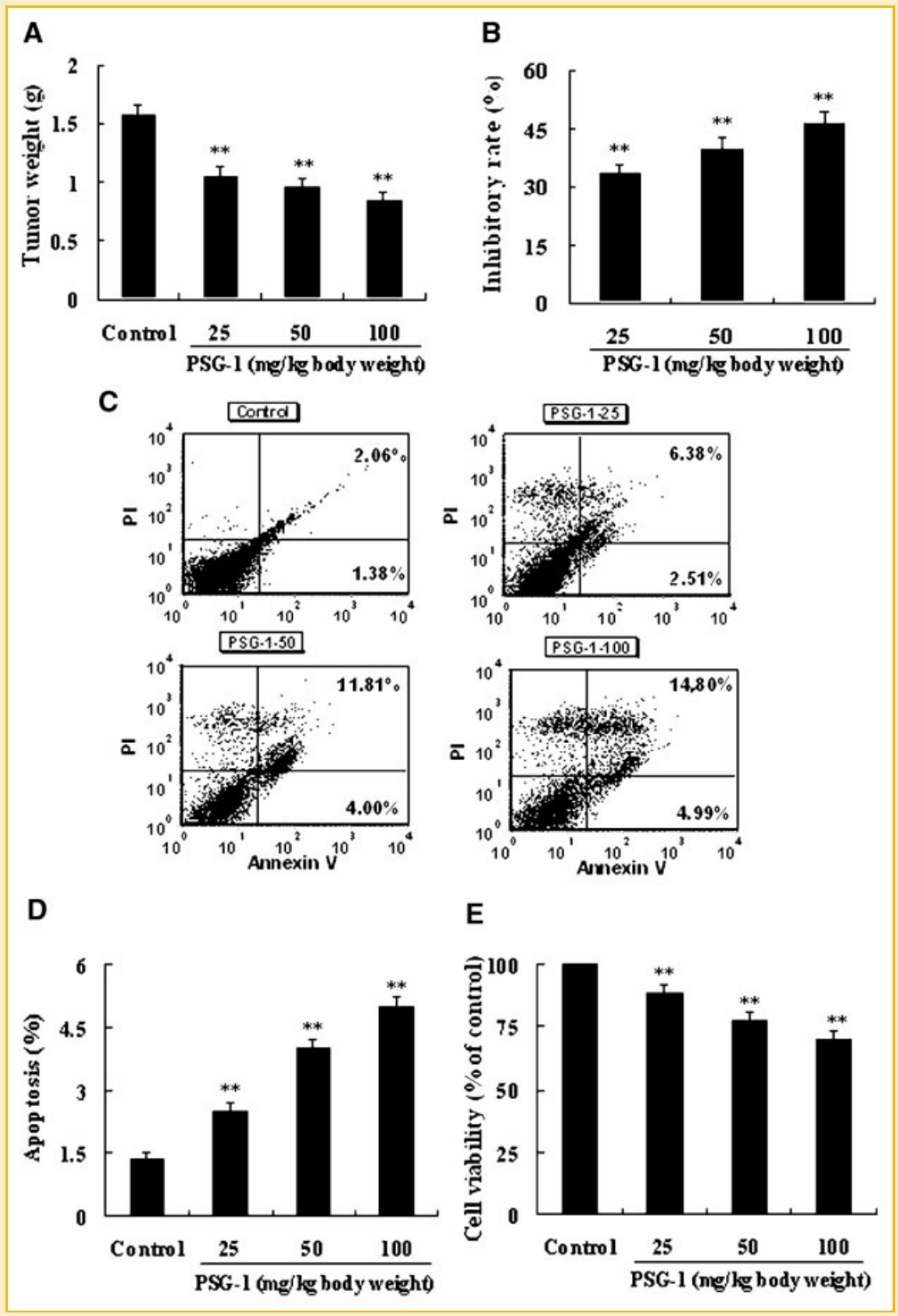


Fig. 1. PSG-1 inhibits the tumor growth of S-180-bearing mice via the induction of apoptosis. A: The tumor weight in S-180-bearing mice. B: The inhibition rate of PSG-1on tumor. C,D: Detection of apoptotic cells by AnnexinV and PI double staining. C: S-180 tumor cells stained with AnnexinV-FITC and PI labeling and analyzed by flow cytometry. D: Column bar graph of apoptosis. E: Cell proliferation was determined by MTT assay. Values are mean ± SEM of eight mice. $^{**}P < 0.01$ versus control group.

of apoptotic signaling. We conducted experiments to analyze ROS production in the tumor of S-180-bearing mice. As shown in Figure 2D,E, PSG-1 caused a rapid and significant increase in DCF fluorescence. These findings indicate that PSG-1-induced apoptosis was associated with the elevation of ROS production.

EFFECTS OF PSG-1 ON CON A OR LPS-INDUCED LYMPHOCYTES PROLIFERATION IN S-180-BEARING MICE

The normal murine lymphocyte proliferation ratio induced by Con A or LPS and treated with RPMI 1640 medium was regarded as 100%. The proliferate responses of lymphocytes to both T-cell and B-cell

TABLE I. Effects of PSG-1 on Immune Function in S 180-Bearing Mice

Group	Thymus index (mg/g)	Spleen index (mg/g)	TNF-α (pg/ml)	IL-2 (pg/ml)	Lymphocyte proliferation rate (%)	
					Con A-induced	LPS-induced
Normal	n.d.	n.d.	n.d.	n.d.	100	100
Control	2.37 ± 0.11	3.60 ± 0.35	19.70 ± 1.20	27.65 ± 1.85	70.48 ± 5.19	76.65 ± 5.06
PSG-1-25	$2.86 \pm 0.15^{*}$	$4.71 \pm 0.48^{**}$	$22.78 \pm 1.98^{*}$	$33.27 \pm 2.02^{**}$	$99.28 \pm 6.94^{**}$	$100.43 \pm 3.59^{**}$
PSG-1-50	$3.32 \pm 0.18^{*}$	$5.51 \pm 0.57^{**}$	$26.83 \pm 2.44^{**}$	$36.48 \pm 1.91^{**}$	$109.75 \pm 5.87^{**}$	$114.98 \pm 6.51^{**}$
PSG-1-100	$3.60 \pm 0.20^{**}$	$5.71 \pm 0.64^{**}$	$27.83 \pm 2.06^{**}$	$38.96 \pm 2.13^{**}$	$111.25 \pm 7.29^{**}$	$118.74 \pm 7.14^{**}$
CTX	$1.66 \pm 0.16^{*}$	$2.88 \pm 0.32^{*}$	$16.76 \pm 1.34^{*}$	$23.94 \pm 2.07^{**}$	$60.98 \pm 5.50^{*}$	$67.57 \pm 3.47^{*}$
PSG-1 + CTX	$3.07 \pm 0.11^{\$}$	$4.81 \pm 0.51^{\$\$}$	$22.60 \pm 1.63^{\$}$	$32.81 \pm 2.22^{\$\$}$	$97.84 \pm 5.94^{\$\$}$	$96.77 \pm 4.80^{\$\$}$

S-180-bearing mice were administered intraperitoneally (i.p.) with the test compounds for 10 days once daily. S180-bearing control group and normal group received the same volume of sodium chloride 0.9% injection. Thymus, spleen indexes, concentration of TNF-α and IL-2 were determined on day 11. Values are expressed as mean ± SEM of eight mice for each group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ compared to CTX group. n.d., not determined.

mitogens (Con A and LPS, respectively) were reduced markedly in control group, compared with normal group (Table I). Meanwhile, when the S-180-bearing mice were administrated with PSG-1, their lymphocyte proliferation activities were increased significantly in S-180-bearing mice group.

EFFECTS OF PSG-1 ON CYTOKINE PRODUCTION IN SERUM OF S-180-BEARING MICE

Effects of PSG-1 on IL-2 and TNF-α level in murine serum were determined by ELISA. As shown in Table I, the IL-2 and TNF-α levels in control group were significantly lower than normal group. At the same time, administration of PSG-1 significantly increased the IL-2 and TNF-α level in serum of S-180-bearing mice.

PSG-1 MARKEDLY ENHANCED THE CTX-MEDIATED ANTI-TUMOR ACTIVITY IN S-180-BEARING MICE

CTX is a mainstay cancer chemotherapy agent, its immunosuppressing activity represents a major clinical challenge and main limiting factor for sustained clinical use. As shown in Table I, CTX resulted in immunosuppressive effects, as evidenced by the decrease of spleen index, lymphocyte proliferation and concentrations of TNF-α and IL-2 in serum. But the PSG-1 could renew it (Table I). Meanwhile, PSG-1 (25 mg/kg body weight) and CTX (20 mg/kg body weight) both showed a lower inhibition rate of S-180 tumor and apoptosis when they were used alone. But when they were mixed at the same dose, the anti-tumor activity was raised rapidly (Fig. 5A,B). We also found that the combination of PSG-1 and CTX caused a significant increase in apoptosis ($AnnexinV^{+}/PI^{-}$), necrosis ($AnnexinV^{+}/PI^{+}$) and antiproliferative effect compared with PSG-1 and CTX treatment alone in the tumor of S-180 bearing mice (Fig. 5C–E). These results suggest that PSG-1 enhanced the anti-tumor activities of CTX by activating host immune response.

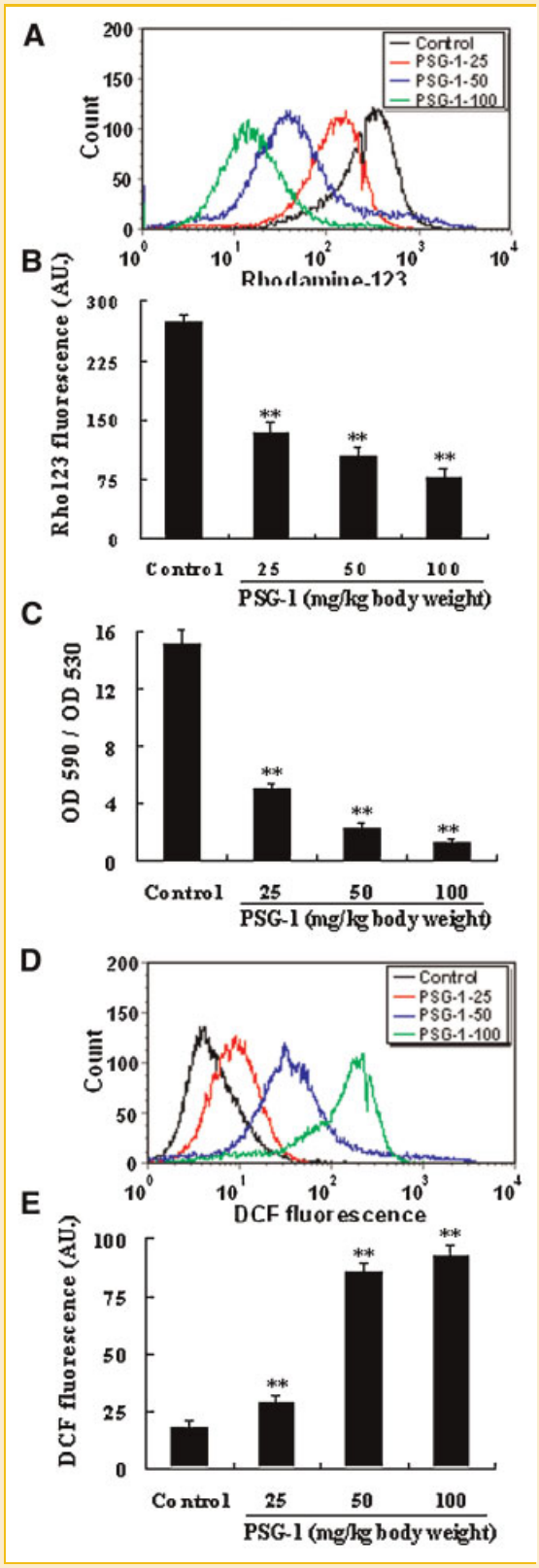
DISCUSSION

Ganoderma atrum has played an important role as an ancient medicine/health food for centuries and there is a growing trend for its medical use. The polysaccharide has been recognized as one of the main active components in *G. atrum*, but there is little information about polysaccharide of *G. atrum* on anti-tumor activity [Chen et al., 2009]. In the present study, we isolated the polysaccharide (PSG-1) from *G. atrum* and investigated its anti-tumor activity using S-180-bearing mice in vivo. Our results indicate that PSG-1 showed a strong anti-tumor effect, as evidenced by the inhibition of tumor growth in S-180-bearing mice.

Cancer is a disease that involves excessive proliferation of cells and abandonment of their ability to die [Croci et al., 2008; Malíková et al., 2008; Pratap et al., 2009]. Normally, cells can kill themselves in a balanced process known as 'apoptosis.' Growing evidence indicates that anti-tumor therapy involves the triggering of tumor cells to undergo apoptosis [Altieri et al., 2009]. In this study, antiproliferative action on murine Sarcoma S-180 and tumor apoptosis are shown during PSG-1-induced anti-tumor effect. Additionally, it has been reported that anti-tumor therapy not only causes apoptosis, but other forms of cell death as well, such as necrosis [Sasi et al., 2009]. A significant increase of necrosis was also shown in PSG-1 treatment groups, indicating that the potential mechanisms of PSG-1 against tumor may be complex.

Apoptosis is a process of gene-mediated programmed cell death for the elimination of unwanted cells in various biological systems and is the key phenomenon in cell death induced by anti-tumor treatment [Aqeilan et al., 2004; Li et al., 2008]. It is now well-established that apoptosis occurs through two major signaling pathways, which are mitochondria-mediated apoptotic pathway and death receptor-mediated apoptotic pathway [Henson et al., 2003]. Currently available data indicate that elimination of malignant cells often depends on mitochondria apoptotic pathway [Ghosh et al., 2006]. More recently, our results have also shown that mitochondrial apoptotic pathway was related to anti-tumor effect in human hepatoma cells [Li et al., 2009]. Therefore, several apoptosis-associated parameters of mitochondrial pathway were investigated in this study.

Studies have shown that disruption of $\Delta\psi_m$ may lead to the release of several apoptogenic proteins, initiating the cascade of events that result in caspase activation during mitochondria-dependent apoptotic pathway [Nakazato et al., 2005]. Flow cytometric studies revealed a very significant depletion of $\Delta\psi_m$ in PSG-1 treatment groups. Mitochondrial dysfunction releases several apoptogenic proteins, most notably cytochrome *c*. The release of cytochrome *c* from the mitochondria into the cytosol was found in the PSG-1 treatment groups. These findings suggest that



PSG-1 induces anti-tumor effect through mitochondrial-mediated apoptosis pathway.

The release of the mitochondrial protein cytochrome *c* precedes the downstream event of caspase activation. Caspases play an important role in the execution of apoptosis. Caspases transduce the apoptotic signal cascade and engage cellular targets leading to apoptosis. Caspase-3, one of the key effectors, might be activated by caspase-9, through the mitochondrial pathway [Giagkousiklidis et al., 2005; Hossain et al., 2009]. Consistent with previous studies, our current results demonstrated that the tumor apoptosis induced by PSG-1 was accompanied by an increase of caspase-9 and -3 activities.

It is well-known that production of ROS is a common mechanism in one of the representative pathways of apoptosis [Liu and Chang, 2009]. In the present study, the results showed that mitochondria were related to PSG-1-induced tumor apoptosis. As the mitochondrial respiratory chain on the inner mitochondrial membrane is a major intracellular source of ROS, we investigated the relevance of ROS generation to apoptosis. The results showed that PSG-1-induced apoptosis in the tumor of S-180-bearing mice was in association with the elevation of ROS production, suggesting that ROS production is related to PSG-1-induced mitochondrial-mediated apoptosis.

The Bcl-2 family, which possesses both anti- and pro-apoptotic members, acts as a checkpoint upstream of mitochondria dysfunction [Tyagi et al., 2006]. Bcl-2 can prevent ROS generation and control the mitochondrial permeability by opposing the effect of Bax, thereby blocking cytochrome *c* release [Chan et al., 2007]. The anti-apoptotic function of Bcl-2 is thought to be primarily derived from Bcl-2 resided in the mitochondria. Under normal conditions, Bax exists as a soluble monomer in cytosol. However, upon stimulation, Bax translocates to mitochondria and the level of mitochondrial Bcl-2 decreases [An et al., 2004; Dai and Grant, 2007]. In this study, Western blot analysis revealed that administration of PSG-1 resulted in the reduction of mitochondrial Bcl-2 and the Bax translocation from cytosol to mitochondria. These results indicate that Bcl-2 family proteins may play a critical role in regulating tumor apoptosis induced by PSG-1.

Our results, together with the results of previous studies, suggest that PSG-1 induce tumor apoptosis through mitochondrial death pathways, as evidenced by the alteration of Bcl-2 family proteins, elevation of ROS production, loss of $\Delta\psi_m$, release of cytochrome *c* and activation of caspase-3 and -9.

An increasing number of studies indicate that many polysaccharides isolated from plants, animals, and microorganisms

Fig. 2. Effect of PSG-1 on the loss of $\Delta\psi_m$ and generation of ROS in the tumor of S-180-bearing mice. A,B: Flow cytometric analysis of $\Delta\psi_m$ as estimated by the Rho 123 fluorescence. A: Flow cytometric analysis of $\Delta\psi_m$ as estimated by the Rho 123 intensity. B: Column bar graph of cell fluorescence for Rho 123. C: Confirmation that PSG-1 caused $\Delta\psi_m$ depolarization by JC-1 staining. The values of OD at 590 and 530 nm were determined by a spectrofluorometry. The $\Delta\psi_m$ was expressed as the ratio of OD590/OD530. D,E: Flow cytometric analysis of ROS generation as estimated by the fluorescence of 2′,7′-dichlorofluorescein (DCF). D: Flow cytometric histograms of DCF fluorescence in cardiomyocytes. E: Column bar graph of cell fluorescence for DCF. Values are mean ± SEM of eight mice. $^{**}P < 0.01$ versus control group.

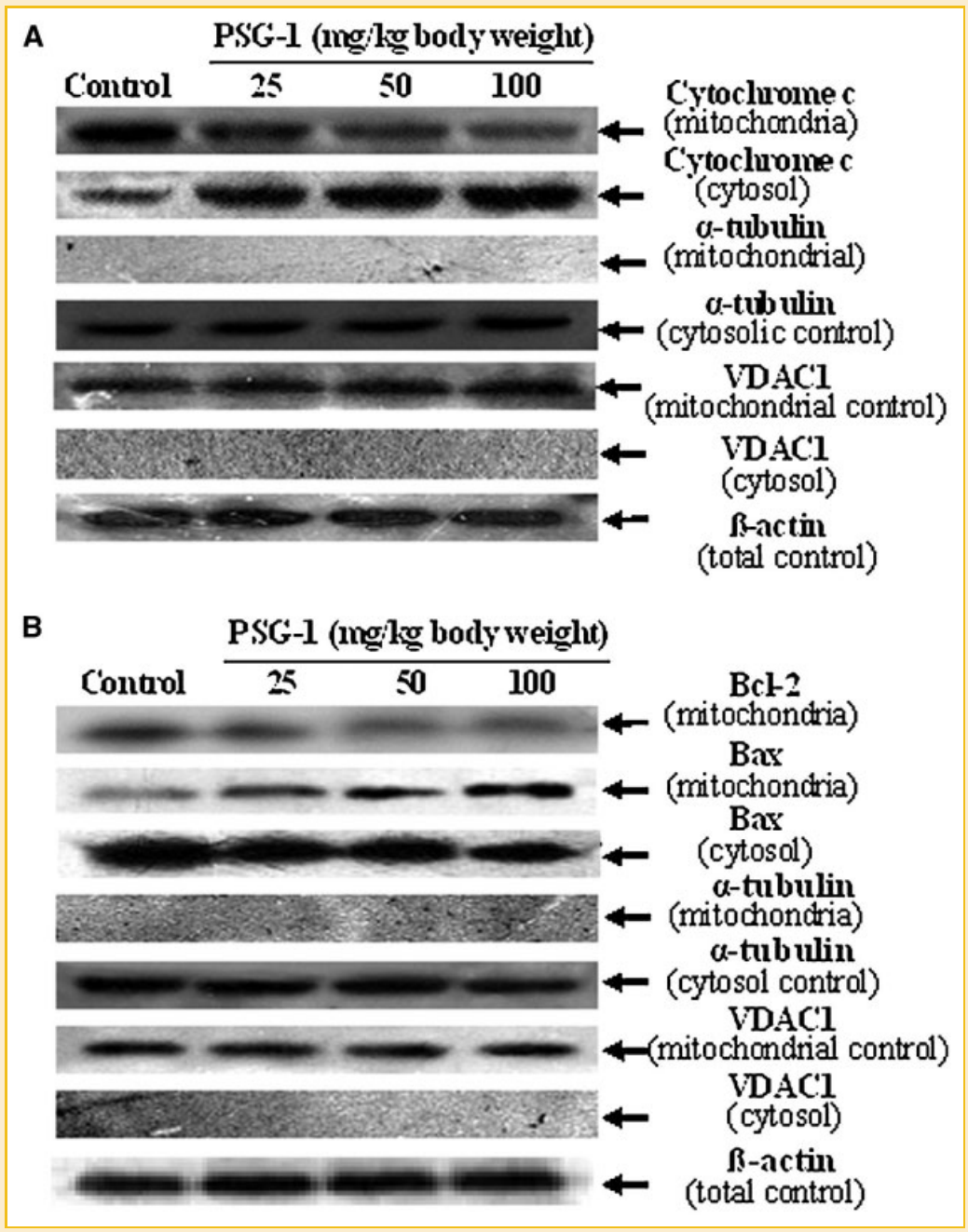


Fig. 3. Effect of PSG-1 on cytochrome *c* release and the expression of Bcl-2 family in the tumor by Western blot analysis. A: The cytosolic and mitochondrial proteins were analyzed by Western blot with anti-cytochrome *c*, anti-α-tubulin, anti-VDAC1, and anti-β-actin antibodies. B: The cytosolic and mitochondrial proteins were analyzed by Western blot with anti-Bax, -Bcl-2, -α-tubulin, -VDAC1, and -β-actin antibodies.

have potent immunotherapeutic properties with respect to the prevention and treatment of cancer [Niizuma et al., 2007]. The occurrence, development and prognosis of cancer are closely related to the immune status of the cancer patients. Further study is thus required to investigate the effect of PSG-1 on immune function in S-180-bearing mice. Immune response of body is composed of specific and non-specific immunity. The specific immune response includes humoral immunity and cellular immunity [Ooi and Liu, 2007]. The humoral defense through antibody response is regulated by B cells and other immune cells involved in antibody production and immunization. Cell-mediated immune defense was mediated specifically by T cells including nature killer cells [Barber and Sentman, 2009]. In this work, T- and B-cell proliferation activity of the control group was significantly lower than normal group. When transplanted S-180 tumor mice were administrated with PSG-1, the T- and B-cell proliferation activities were increased significantly than control group. The thymus and spleen are two important immune organs. The thymus and spleen indexes were

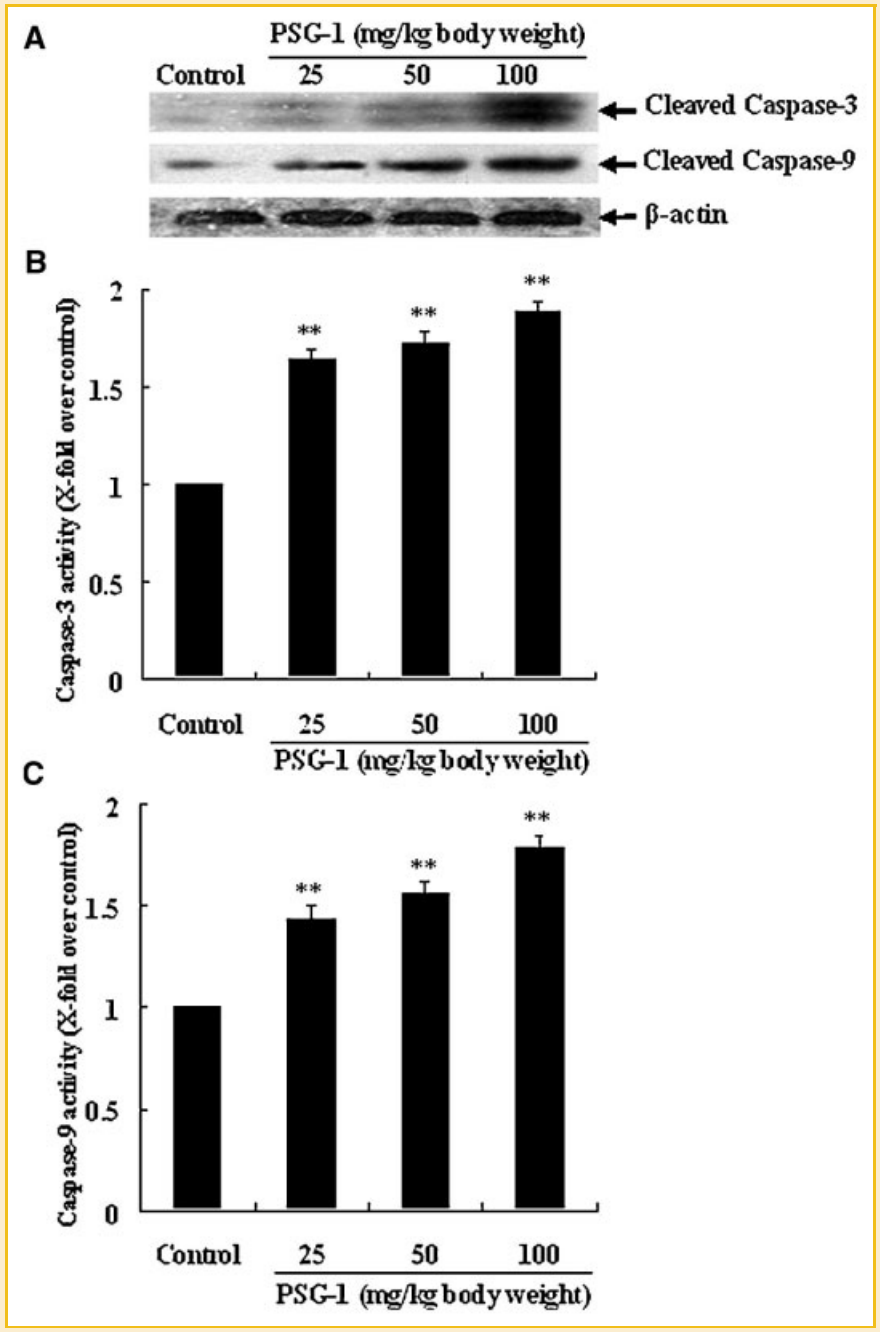


Fig. 4. Effect of PSG-1 on the activation of caspases in the tumor of S-180-bearing mice. A: The proteins were analyzed by Western blot with anti-cleaved capase-3, anti-cleaved caspase-9 and anti-cleaved-β actin antibodies. B,C: Quantitative analysis of caspase-3 and -9 activities in the tumor of S-180-bearing mice. Values are mean ± SEM of eight mice. $^{**}P < 0.01$ versus control group.

important index for non-specific immunity [Kim et al., 2001]. The thymus and spleen were markedly increased in S-180-bearing mice treated with PSG-1 compared with control group. Cytokines are soluble glycoproteins that are crucial in the induction and regulation of immune response. IL-2 and TNF-α are two important cytokines mediating the immune response. IL-2 produces a significant activity through the receptor system with distribution in the T cells, B cells, NK cells, and monocytes [Xu et al., 2008]. TNF-α is a cytokine with anti-tumor and immunomodulatory properties. It has been recognized as an important host defense cytokine that affects tumor cells [Terlikowski, 2002; Dhanji and Teh, 2003; Yuan et al., 2006]. In the present study, both IL-2 and TNF-α levels in PSG-1 test group were significantly higher than control group. In a word, these data suggest that PSG-1 induces antineoplastic action of S-180-bearing mice related to activation of host immune response.

CTX is the most widely used alkylating agent in cancer chemotherapy to date. The anti-tumor effect of CTX is in proportion

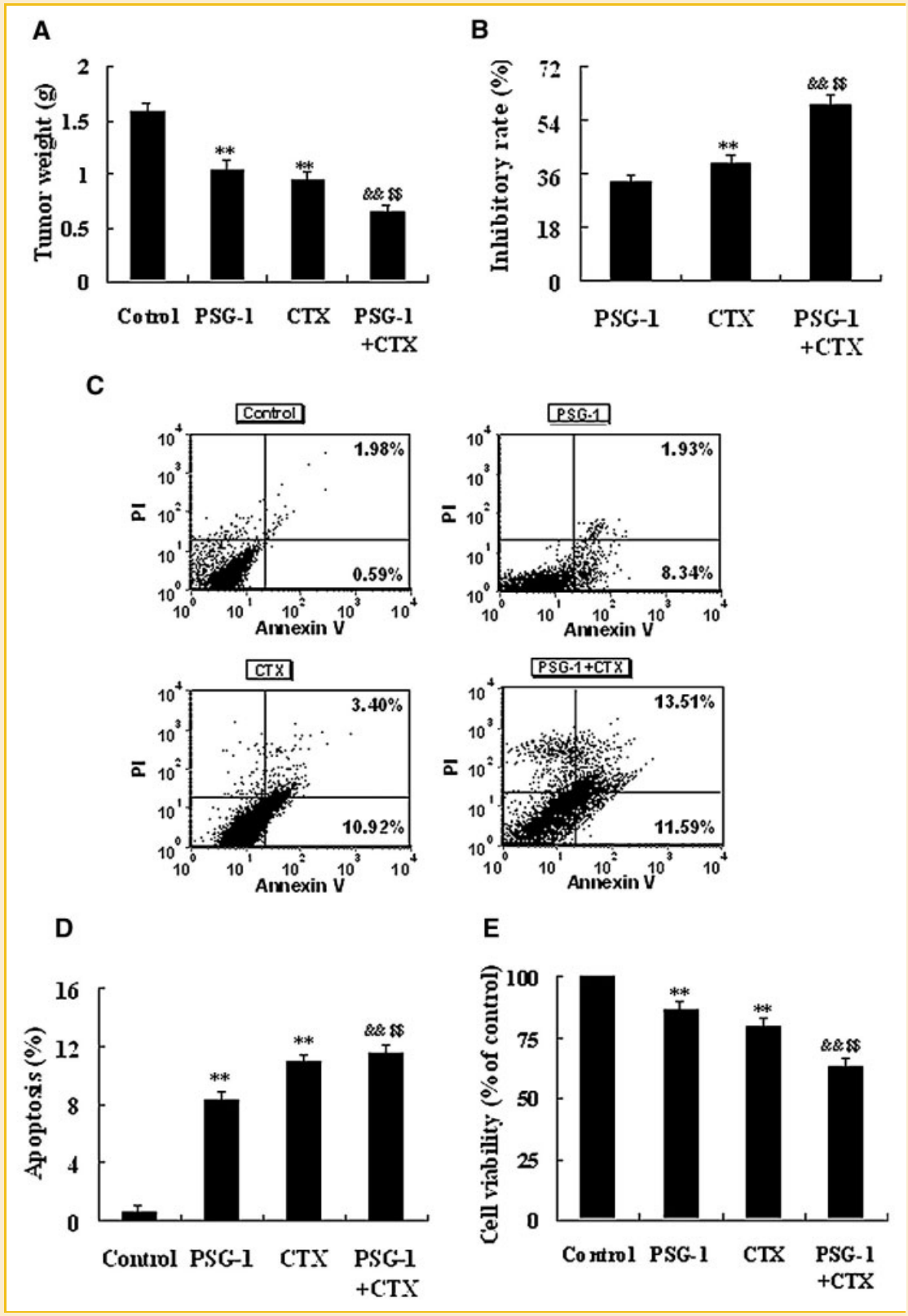


Fig. 5. PSG-1 potentiates CTX-mediated anti-tumor activity in S-180-bearing mice. A: The tumor weight in S-180-bearing mice after PSG-1 (25 mg/kg body weight) treatment with or without CTX (20 mg/kg body weight). B: The inhibition rate of PSG-1 or association with CTX on tumor. C,D: PSG-1 enhanced CTX-mediated apoptosis in S-180 tumor cells (C) S-180 tumor cells stained with AnnexinV and PI labeling and analyzed by flow cytometry. D: Column bar graph of apoptosis. E: Cell proliferation was determined by MTT assay. Values are mean ± SEM of eight mice. $^{**}P < 0.01$ versus control group, $^{\$\$}P < 0.01$ compared to CTX group, $^{\&\&}P < 0.01$ compared to PSG-1 group.

to the dose of CTX administered, often resulting in immunosuppressive effects [Aneja et al., 2007; Segers et al., 2008]. Our results have also shown that spleen index, T- and B-cell proliferation and concentrations of IL-2 and TNF-α in the CTX group were significantly decreased compared with the control group. Meanwhile, the PSG-1 could restore the immunity of mice, which was depressed by CTX. Additionally, we found that the combination of CTX and PSG-1 resulted in a significant increase in anti-tumor activity compared with CTX or PSG-1 treatment alone in S-180-bearing mice. These findings imply that PSG-1 increases the anti-tumor activity of CTX by regulating the immune system.

CONCLUSIONS

Taken together, the present study has for the first time provided unequivocal evidence that polysaccharide from *G. atrum* (PSG-1) possesses potent anti-tumor activity. In this study, the most important observations were that PSG-1 markedly suppressed the tumor growth, and induced tumor apoptosis through mitochondrial pathways, and these effects may be achieved through modifying the immune function in S-180-bearing mice. Furthermore, the combination of PSG-1 and chemotherapeutic agent (CTX) may provide a new strategy to enhance therapeutic activity. In conclusion, PSG-1 may have potential as a novel therapeutic agent to replace or augment more cytotoxic agents currently used to treat the carcinoma patients.

ACKNOWLEDGMENTS

The financial supports for this study by the National High Technology Research and Development Program of China (863 Program; 2008AA10Z325), and Objective-Oriented Project of State Key Laboratory of Food Science and Technology (SKLF-MB-200806) and Key Science and Technique Project of Jiangxi Provincial Department of Science, are gratefully acknowledged.

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