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Novel highly branched water-soluble heteropolysaccharides as immunopotentiators to inhibit S-180 tumor cell growth in BALB/c mice

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ABSTRACT

The *in vitro* antitumor activities and *in vivo* immunomodulatory effects of water-soluble highly branched heteropolysaccharides isolated from *Rhizoma panacis Japonici* were evaluated by three cell lines and BALB/c mice implanted with Sarcoma 180 (S-180) tumors. The heteropolysaccharides showed potent tumor therapeutic effect by potentiating the animal's immune responses including an increase in white blood cell count and lymphocyte number. The heteropolysaccharides also induced apoptosis in the S-180 cells and alleviate the short-term side effect of cisplatin (DDP) to the animal during the treatment period. The bioactivity of the heteropolysaccharides might be due to their unique structures that had an α-(1 → 4)-D-glucan main chain with side chains containing mannose and galactose residues, a spherical chain conformation, as well as high water solubility, all of which facilitated their interaction with the surface receptors of the immune cells.

1. Introduction

Polysaccharides are the third kind of macromolecules carrying essential biological information following nucleic acids and proteins. They play an important role with their structural diversity in living organisms in almost every aspect of physiological events, similar to nucleic acids and proteins (Chen et al., 2008; Hirabayashi, 2004). Since over half of all proteins are glycosylated; this makes polysaccharides one of the most common post-translational modification motifs (Ding, Lopez-Burks, Sanchez-Duran, Korc, & Lander, 2005; Galanski & Keppler, 2007; Pilobello, Krishnamoorthy, Slawek, & Mahal, 2005; Raman, Sasisekharan, & Sasisekharan, 2005; Smetsers et al., 2003; Tocilj et al., 2008). Engelhorn et al. (2006) have showed that immunity is enhanced by protein–polysaccharide complex that promotes T helper cell responses and the presentation of a vast repertoire of antigen variants to the immune system. The polysaccharide from *Phellodendron Chinese Schneid* showed tumor inhibitory activities by directly acting to immune system as well as increasing the number of circulating blood leukocytes and total peritoneal exudate cells (Park, Lai, & Kim, 2004). Water-soluble polysaccharides from the mycelium of a medicinal mushroom *Ganoderma tsugae* with a structure of heteropolysaccharide–protein complexes composed of (1 → 3)-β-D-glucans and (1 → 4)-α-D-glucans, had significantly stronger antitumor activity against solid tumor Sarcoma 180 (S-180) (Peng, Zhang, Zeng, & Kennedy, 2005). A water-soluble heteropolysaccharide from an edible mushroom, *Poria cocos*, exhibited potent *in vivo* antitumor activity against S-180 tumors, (Zhang, Chen, Xu, Zeng, & Cheung, 2005). Three neutral proteoglycans derived from the mushroom mycelium of *Pleurotus ostreatus* inhibited tumor cell proliferation, elevated natural killer (NK) cell cytotoxicity and nitric oxide production in murine macrophages (Sarangi, Ghosh, Bhutia, Mallick, & Mait, 2006). Mushroom polysaccharides could inhibit tumor growth by inducing apoptosis and enhancing the host's immunity (Chauhan et al., 2005; Moradali, Ghods, & Hedjaroude, 2007; Zhu et al., 2007). Conventional chemotherapy or combination of radio- and chemotherapy for cancer patients usually has a low survival rate due to its detrimental effect on the patient's normal cells (Hahn & Weinberg, 2002; Thies, Dautel, Meyer, Pfüller, & Schumacher, 2008). An understanding of the complex process of how polysaccharides can affect neoplastic transformation and immune response in humans may provide an alternative approach to cancer treatment.

Rhizoma panacis Japonici (RPJ), a Chinese traditional medicine, has been used as a substitute for *Panax ginseng* whose root is now widely used for many diseases. The therapeutic efficacy of ginseng root extract has not yet been fully established and comprehensive

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clinical data in humans are still lacking because of the poor quality of most clinical trials on ginseng (Xiang, Shang, Gao, & Zhang, 2008). In our previous work, five water-soluble polysaccharides coded as RPS1, RPS2, RPS3, RPS4 and RPS5 extracted from RPJ with different aqueous medium have been found to be a highly branched heteroglycan with a main chain of α-(1 → 4)-D-glucan having galactose, arabinose, mannose and/or xylose residues in the side-chain. The molecular mass of these heteropolysaccharides was in the range of 3×10^4–1×10^6 Da and they all adopted a spherical conformation in water (Huang & Zhang, 2009; Huang, Huang, Li, & Zhang, 2009). Our previous studies had indicated that these RPSs could inhibit the *in vitro* growth of human hepatocarcinoma (HepG2) tumor cells but were non-toxic to normal African green monkey kidney fibroblast cells (COS-7) (Huang, Ren, Duan, & Zhang, 2010) in a way similar to the antitumor β-(1 → 3)-D-glucans from mushrooms (Tao & Zhang, 2006; Tao, Zhang, & Cheung, 2006; Tao, Zhang, Yan, & Wu, 2007; Zhang, Cui, Cheung, & Wang, 2007).

Sarcoma 180 tumor is an allogenic tumor derived from the murine fibrosarcoma cell line and is commonly used as a transplantable animal tumor in mouse (Dunham, 1953). In this work, the potential therapeutic effects of RPS were evaluated by the *in vitro* and *in vivo* inhibition of S-180 tumor cells. The structure–activity relationships between the structural characteristics of RPS including the side-branching, the molecular mass and the spherical chain conformation of the heteropolysaccharides with their tumor growth inhibition and immunopotentiation, were discussed.

2. Experimental

2.1. Materials

Sarcoma 180 (S-180) cells and African green monkey kidney fibroblast cells (COS-7) were obtained from ATCC (USA). The two cell lines were authenticated by morphological characteristic check under microscope and freshly cultured at third-passage in the laboratory. Lymphocytes were separated from the spleens of BALB/c mice and cultured directly. Six to eight-week-old male and female BALB/c mice (weight: 20 ± 2 g) were obtained from the Animal Center, Wuhan University, Wuhan, China.

Rhizoma panacis Japonici (RPJ) was cultivated in Enshi, Hubei Province, China. The water-soluble heteropolysaccharides (RPSs) used in this study were obtained from RPJ by protocols described previously (Huang & Zhang, 2009). 3-(4,5)-Dimethylthiazol-2,5-diphenytetrazolium bromide (MTT) was purchased from Invitrogen Corporation (USA). 5-Fluorouracil (5-FU), lipopolysaccharide (LPS), concanavalin A (Con A) were purchased from Sigma–Aldrich (China). Cisplatin (DDP), Lentinan (LTN), phycoerythrin (PE) anti-mouse CD8a and fluorescein isothiocyanate (FITC) anti-mouse CD4 kit and ABC hemolytic agent were purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). All chemical reagents were commercially available and used without further treatment.

2.2. In vitro MTT assay

The heteropolysaccharide samples were dissolved in normal saline (NS, 0.9% NaCl). The cells were incubated with the samples at doses from 2 μg/ml to 200 μg/ml for the S-180 cell line, 0.16 μg/ml to 500 μg/ml for the COS-7 cell line, and 1 ng/ml to 100 μg/ml for lymph cell line for 48 h in 96-well culture plates at 37 °C. After the addition of MTT reagent, the cells were left for 4 h before the cell numbers were quantified by a microplate reader (GE Nios VA200, Tecan, Austrian) with optical density (*OD*) measured at an absorbance of 570 nm. The S-180 *in vitro* antitumor activities of the tested samples were expressed as: Inhibition percentage (%) = $[(OD_{cell} - OD_{sample})/(OD_{cell} - OD_{blank})] \times 100\%$. Immunostimulatory activities expressed as the lymphocyte stimulator index (*SI*) and the relative cell viability of COS-7 were calculated as: *SI* or cell viability (%) = $(OD_{sample} - OD_{blank})/(OD_{cell} - OD_{blank}) \times 100\%$, where OD_{sample} was the *OD* for cells treated with the heteropolysaccharide or control, and OD_{blank} was *OD* for RPMI 1640 culture medium contained 2% calf blood serum, OD_{cell} was *OD* for cells without the treatment of heteropolysaccharides, respectively. 5-FU was used as positive control in S-180 *in vitro* antitumor activities and as negative control in cell viability of COS-7. Con-A was used as positive control and LPS as negative control in the measurement of immunostimulatory activities.

2.3. In vivo assay

BALB/c mice were housed under standard conditions of temperature (25 ± 2 °C), relative humidity (55% ± 10%) and 12 h light/12 h dark cycle at the Animal Laboratory of College of Pharmacy, Wuhan University, Wuhan, China. Sarcoma S-180 cells adjusted to 1×10^7 cells/ml in NS were inoculated in the animals by intraperitoneal (i.p.) injection at a dose volume of 0.1 ml/10 g body weight of the animal. Different treatments including RPSs, positive control (DDP and Lentinan) were applied to individual animal groups when the tumor formed on the fourth day after tumor implantation shown in Table 1. DDP was administrated by intraperitoneal injection (i.p.); RPSs and Lentinan were fed by oral feeding. Both intraperitoneal and oral administration of the samples was at a dose volume of 0.1 ml/10 g body weight of the animal at different concentrations (4, 10 and 20 mg/kg). After 7 days of treatment, the surviving mice were sacrificed by cervical dislocation. Their blood was collected and the tumor ascites were excised and weighed. Visceral organs including liver, spleen, kidney and thymus of the animals were removed and weighed. The *in vivo* tumor inhibition ratio was calculated as: Inhibition ratio (%) = $100((A - B)/A)$, where *A* is the average tumor weight of the control group and *B* is the tumor weight of treatment group. The body weight ratio was calculated as the average ratio of the final-to-initial body weight of the animal group. The viscera indices were calculated as the average weight ratio of the viscera to the body (mg/g) in the animal group. The hematological and biochemical parameters of the animal's blood were determined by standard protocols used for human's blood with a Burker hemocytometer.

2.4. CD4 and CD8 expressions

Flow cytometry (Epics AlttraII, Beckman Coulter, USA) was used to determine CD4 and CD8 expressions on lymphocytes in the animal's blood. Direct labeling of antibodies consisting of fluorescein isothiocyanate (FITC) for determining CD4 expressions and phycoerythrin (PE) for CD8 were used. The lymphocytes were left for 15 min under protecting form light for surface staining of blood cell suspensions at room temperature after red blood cells were lysed by ABC hemolytic agent, washed by PBS and separated at 1000 rpm for 10 min (Gorczyca et al., 2002).

2.5. Morphologies of tissues and cells

Liver, spleen, and kidney were fixed with 10% formalin and the metallographs of the H&E staining tissue slices were taken by an optical microscope (XSP-11CD, Shanghai Caikon Optical Instrument Co. Ltd. China) housed with a digital camera (A95, Canon, Japan). The tumor ascites were centrifuged and fixed with 2.5% glutaraldehyde and the ultra-microcuts were stained with osmic acid. The cell morphology was observed by a transmission electron

Table 1
Tumor inhibition and biochemical parameters from the blood of treated and control groups of BALB/c mice.[#]

Group	Treatment	Dose (mg/kg × days)	Survival/test mice	Inhibition ratio (%)	Weight ratio (%)	Spleen index (mg/g)	Thymus index (mg/g)	ALT (U/L)	AST (U/L)	Urea (μmol/L)	Crea (μmol/L)	WBC (10^9/L)	Lym (10^9/L)
Normal	NS	10 × 7	8/8	–	110.4	3.3	2.5	38	123	6.2	22.4	4–10	0.8–4
S-180 model	NS	10 × 7	7/8	–	122	9.5	0.5	257	650	5.1	21.3	5.2	0.4
Cisplatin	DDP	4 × 7	4/8	100	70.5	2.6	0	578	1794	14.1	40.2	8.2	0.3
LTN	Lentinan	20 × 7	5/8	25.2*	129.3*	7.5*	0.5*	160*	650*	6.2*	18.0*	33.5*	0.7*
LTN + DDP	LTN + DDP	20 × 7 + 4 × 3	5/8	100	105.8*	2.1	0	178*	738*	6.6*	19.1*	10.3	0.3
RPS1	RPS1	20 × 7	6/8	89.4	116.7*	10.7*	0.3*	150*	250*	7.0*	22.0*	18.1*	0.7*
RPS1 + DDP	RPS1 + DDP	20 × 7 + 4 × 3	5/8	100	74.8*	1.7	0.1	185*	391*	8.4*	28.1*	5.8	0.5*
RPS5	RPS5	20 × 7	7/8	31.4*	82.8*	7.4*	0.7*	120*	200**	5.1*	17.0*	44.3*	0.7*
RPS5 + DDP	RPS5 + DDP	20 × 7 + 4 × 3	8/8	100	77.7*	2.1	0.1	164*	308*	7.4*	18.3*	8.1	0.8*

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cre, creatinine; DDP, cisplatin; Lym, lymphocyte; NS, normal saline; WBC, white blood cell.
[#] Mean values with $n = 3$.
* Significant different with cisplatin (DPP) group by Student's t test; $P < 0.05$.

microscope with a magnification of 1200× (TEM, JEM-100CX11, JEOL, Japan).

2.6. Statistical analysis

Results are expressed as the means with standard deviations listed as error bars in the figures. Statistical analysis was performed by using Student's t-test with $P < 0.05$ deemed as statistically significant. All experiments were repeated at least in triplicate unless otherwise stated.

3. Results and discussion

3.1. In vitro antitumor effects

All RPSs demonstrated a dose-dependent inhibition on the growth of S-180 (Fig. 1a). At a concentration of 20 μg/ml, RPS1, RPS2, RPS3 and RPS5 had a growth inhibition percentage of S-180 cells comparable the positive control, 5-Fluorouracil (5-FU) (Fig. 1a). Even a low concentration of 2 μg/ml, all the RPSs except RPS4 had an inhibition percentage above 30. These results suggested the potency of RPSs on the *in vitro* antiproliferation of S-180 cells.

The cytotoxic effect of RPSs on the viability of COS-7 cells at doses from 0.16 to 500 μg/ml is shown in Fig. 1b. In general, the % cell viability of COS-7 cells treated with RPSs was higher than that of 5-FU, the differences were most significant at the highest concentration of 500 μg/ml. This indicated that PRSs were less cytotoxic to normal cell like COS-7 compared to 5-FU and suggested the selective inhibitory effect of PRSs on S-180 cells.

Lymphocytes treated with 100 ng/ml of RPS1 and RPS5 had a higher stimulation index (*SI*) of lymphopoiesis than that of the positive control ConA at 5 μg/ml (Fig. 1c). At 1 ng/ml, RPS1 had a comparable SI% to that of Lentinan which is known to be a potent immunomodulator (Fig. 1c). These results suggested that the immunomodulatory potency of RPS1 and RPS5 were higher than the other RPSs.

3.2. In vivo antitumor effects

The above *in vitro* results suggested that the antitumor effects of RPSs could probably be mediated by immunopotentiation of lymphocytes and direct cytotoxicity to tumor cells with effect on normal cells. RPS1 and RPS5 being the most potent heteropolysaccharides based on their *in vitro* antitumor effects were used in the *in vivo* experiments to further investigate their mechanism of actions.

Table 1 lists the survival rate tumor inhibition ratio and final-to-initial body weight ratio of the different treatment and control groups of mice. The survival rate of mice treated with RPS1 and RPS5 alone was significantly higher than that of cisplatin (DDP) treated mice ($P < 0.01$). Those treated with 20 mg/kg of RPS5 in combination with DDP had a 100% survival rate which was the same as the normal control group without S-180 tumor implantation. RPS1 and RPS5 when applied in conjunction with DDP completely inhibited the tumor growth while RPS5 alone could have a high tumor inhibition ratio of 89.4%. The body weight ratio of the mice treated with RPS1 and RPS5 were significantly higher ($P < 0.01$) than that with DDP. It was obvious that RPS1 and RPS5 reduced the body weight loss and mortality of the animal when compared with DDP which is a widely used clinical anticancer drug (Li et al., 2008). These results were consistent with those from the *in vitro* experiments in that RPSs could have selective antitumor effect against S-180 cells with little harmful side effect to the animals. It was noted that the inhibition ratio of Lentinan, a well known immunopotentiator was only 25.2%. It seemed that RPS1 was the most potent antitumor agent

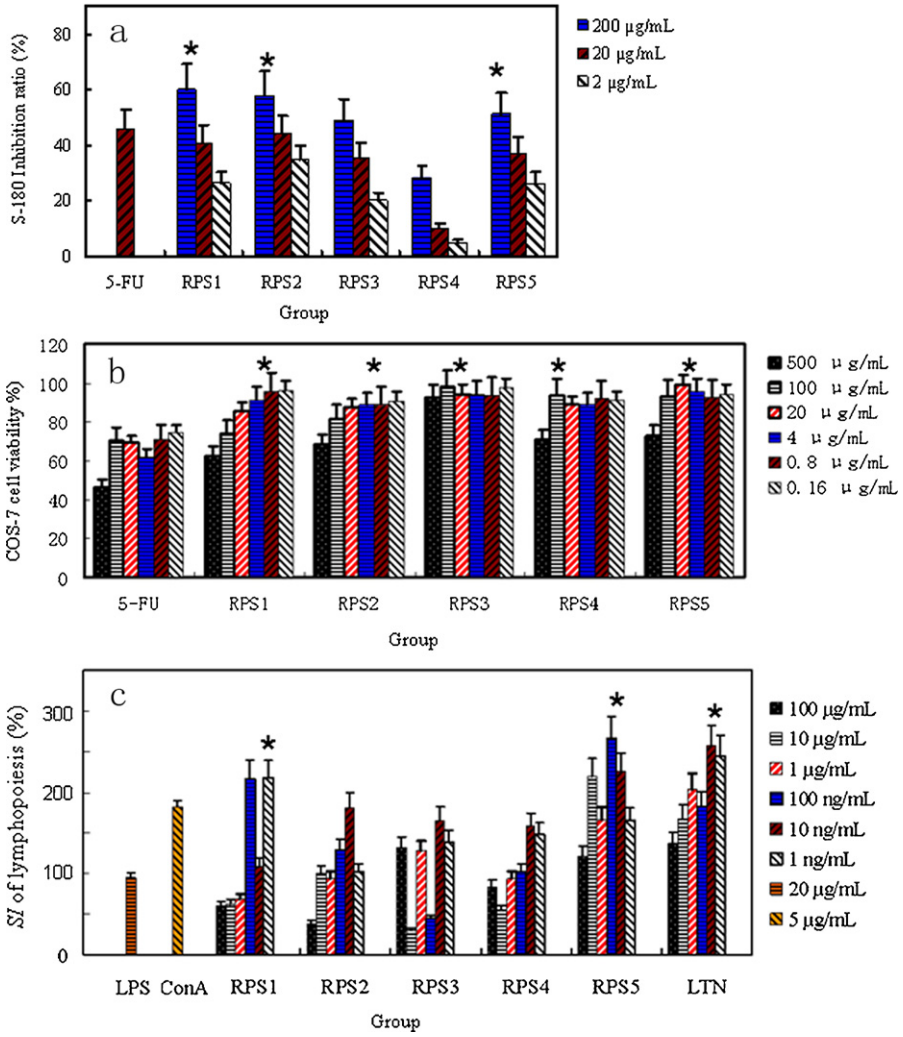

Fig. 1. *In vitro* biological effects of RPSs on three cell lines. (a) Inhibition of proliferation of S-180 cells; (b) COS-7 cell viability; (c) stimulation index (*SI*) of lymphopoiesis. Data are means with error bars representing standard deviations ($n = 3$).

based on its high tumor inhibition ratio and final-to-initial body weight ratios.

3.3. Protection to spleen, thymus, kidney and liver

Both the spleen and thymus indices for Lentinan, RSP1, and RPS5 groups were significantly higher ($P < 0.01$) than those of the DDP. These results were in good agreement with the *in vitro* results on lymphopoiesis (Fig. 1c). The levels of alanine aminotransferase (ALT), aspartate aminotransferase (ALT), urea, and creatinine in all the RPS treatment groups (either on its own or in combination with DDP) were all significantly lower than those of the DDP group (Table 1). Since high levels of AST/ALT and creatinine/urea indicate the malfunctions of liver and kidney, these results suggested that RPS1 and RPS5, either applied alone or in combination with DDP to the animals could enhance the efficacy of the animal's liver and kidney to reduce the toxic side effect of DDP. Similarly, the white blood cell and lymphocyte counts in all the RPS groups were also significantly higher ($P < 0.01$) than those of the DDP group and these results were consistent with the thymus and spleen indices mentioned earlier (Table 1). This suggested that RPS1 and RPS5 could boost up the immune functions of the animal by stimulating the proliferation of immune cells.

The typical appearance of the livers for the RPS5 group and DDP group are shown in Fig. 2a and b, respectively. The liver for the animal treated with RPS5 had a bright red appearance typical of a normal liver, while the one for the DPP treatment group was pale in color and had portions of the liver with signs of fibrosis (indicated by dark arrow in the figure).

Fig. 3 shows the metallographs of slices of liver, kidney and spleen tissues from the four animal groups including the normal (Fig. 3a–c), RPS5 (Fig. 3d–f), S-180 (Fig. 3g–i), and DPP (Fig. 3j–l). From the metallographs, organ tissues of both the normal and RPS5 treatment groups appeared to be normal while those from DDP and S-180 treatment groups showed indications of inflammation and fibrosis, especially in the liver tissues where black spots and loose hepatocytes could be found (Fig. 3j–l). These observations further indicated that RPS5 could protect the vital internal organs of the animals such as liver against fibrosis and minimize the harmful side effects of DDP treatment in mouse implanted with S-180 tumor.

3.4. Stimulation of lymphopoiesis

Helper T cells, also known as CD4+ T cells can excrete cytokines and express CD4 molecules on their surface. They are essential in determining B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes. Cytotoxic T cells, also known as CD8+ T cells, express CD8 on their surface. They are capable of inducing the death of tumor cells as well as somatic cells infected with viruses

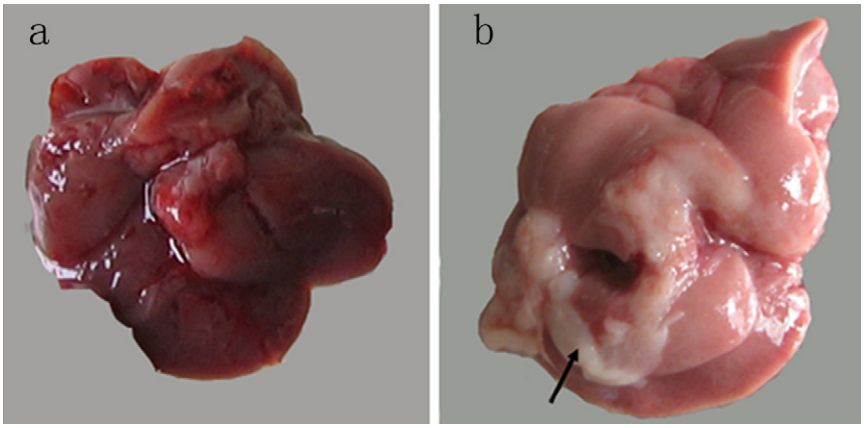

Fig. 2. The typical image of the liver of mice treated with (a) RPS5 and (b) DDP under optical microscope.

(Varki et al., 2009). In order to find out the mechanism by which the heteropolysaccharides could stimulate the immune cells, the CD4 and CD8 expressions on lymphocytes were tested. Fig. 4 shows the flow cytometric analytic results for CD4 with FITC and CD8 with PE staining on T cells in the animal's blood. Compared with the normal control (51 and 12% for CD4 and CD8, respectively as shown in Fig. 4a), the number of CD4 T cells in the RPS1 combined with DDP group was increased significantly by 8% (Fig. 4f), while the number of CD8 T cells was increased significantly by 4 and 11% in the RPS5 group and RPS5 combined with DDP group, respectively (Fig. 4g and h). The numbers of both CD4 and CD8 T cells were decreased significantly by 40 and 6%, respectively in the S-180 group (Fig. 4b), while only CD8 T cell was decreased significantly by 8% in the DDP group (Fig. 4c). The numbers of both CD4 and CD8 T cells were also decreased significantly by 20 and 2%, respectively in the Lentinan group (Fig. 4d) but the % decrease was only half of those found in the S-180 group. There was a small increase in the number of CD8 T cells (2%) but a large decrease (16%) in the number of CD4 T cells found in the RPS1 group (Fig. 4e). These results suggested that RPS1 could mainly activate Helper T cells while RPS5 could stimulate cytotoxic T cells to exert their immunomodulatory effects. It was noted that the *in vivo* T-cell stimulated effects of both RPS1 and RPS5 were significantly stronger than those of Lentinan.

These results could be used to explain the differences observed in the inhibition of S-180 tumor by RPS1 and RPS5 shown in Table 1. From the above results, it was noted that RPS1 killed the tumor cells directly while RPS5 inhibited tumor growth by increasing the immune responses of the animals. These showed that not only cytotoxic CD8+ T cells but also CD4+ T cells can be redirected to trigger lysis of cancer cells at very low effector-to-target ratio, reported previously (Elmir, Casanova, Betbeder, & Triebel, 2001; Baeuerle & Reinhardt, 2009).

3.5. Inducing S-180 cells apoptosis

Apoptosis is a kind of programmed cell death modulated by anti-apoptotic and pro-apoptotic effectors that are considered as targets of anticancer therapy (Baea, Janga, & Jinb, 2006). Morphological

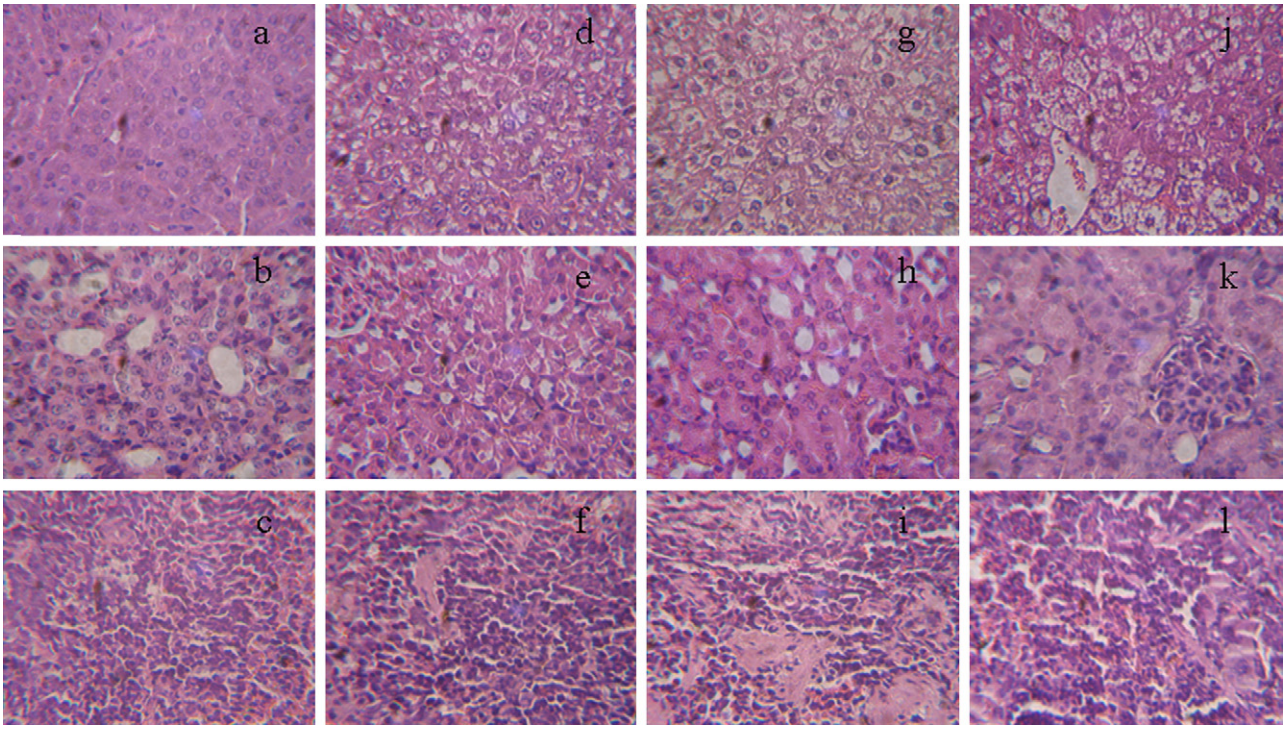

Fig. 3. The metallographs of organ tissues from the liver, kidney, and spleen of mice from the control (a–c); the RPS5 (d–f); the S-180 (g–i); and the DDP groups (j–l).

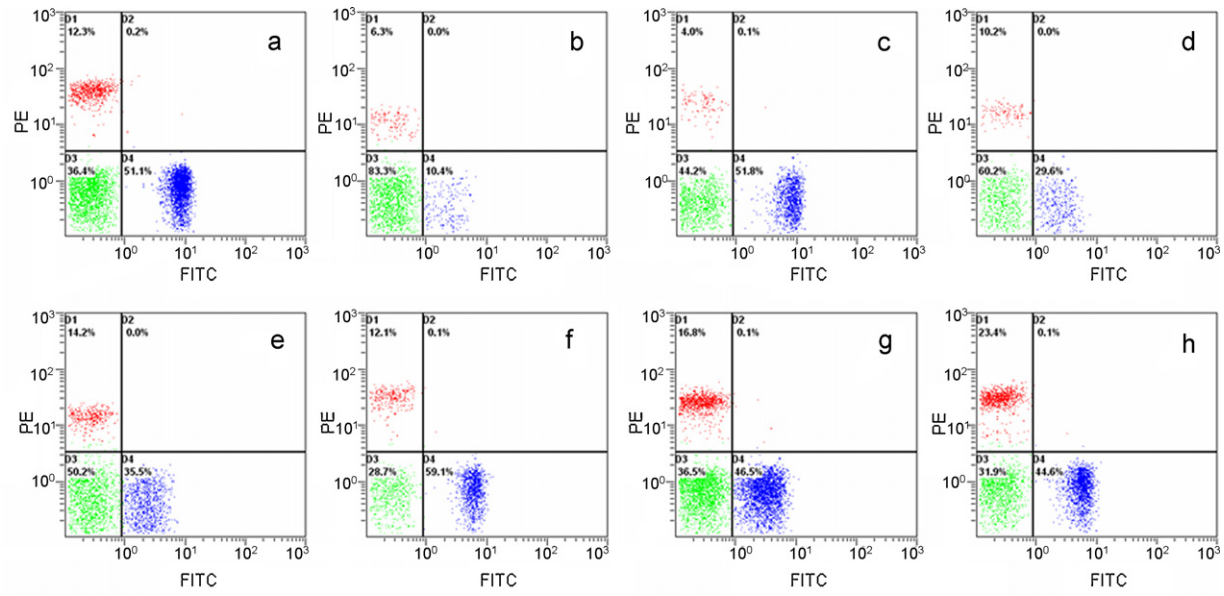

Fig. 4. Flow cytometric analysis with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) staining for CD4 and CD8 of T cells in the blood of mice from (a) the normal; (b) the S-180; (c) the DDP; (d) the Lentinan; (e) the RPS1; (f) the RPS1 and DDP; (g) the RPS5 group; and (h) the RS5 and DDP groups.

changes can provide strong evidence for recognizing the apoptotic process. Fig. 5 shows the morphology of S-180 cells in ascites of the treated mice by TEM. The cells of S-180 group had a normal oval and smooth cell nucleus and cell membrane (Fig. 5a) while the TEM images of the other groups showed some morphological features typical to those of apoptosis. Those cells treated with RPS1 and RPS5 exhibited irregular aggregation of chromatin in the nucleus of the cell (Fig. 5c and d, respectively) and those cells treated by DDP showed deformation in the cell nucleus and cell membrane (Fig. 5b). It is known that changes in cellular morphology, including cell shrinkage, condensation and fragmentation of nuclei are indicative of apoptosis. The morphological details shown in the

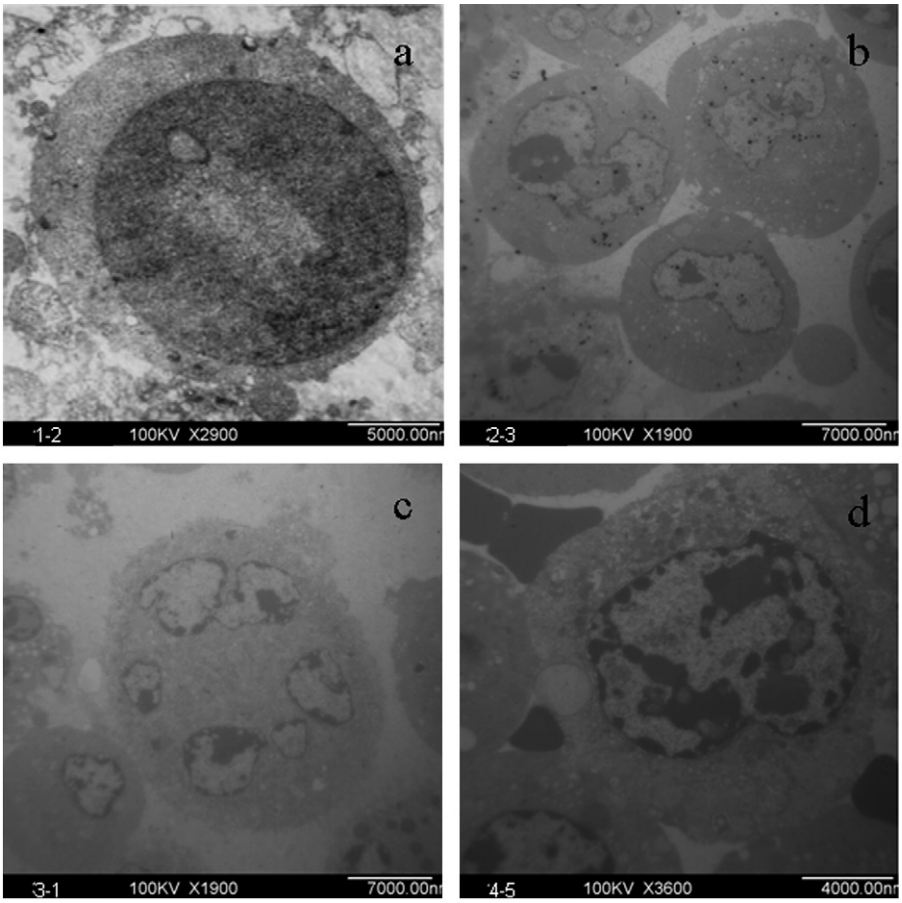

Fig. 5. TEM images of S-180 cells from the mice of (a) the S-180; (b) the DDP; (c) the RPS1; and (d) the RPS5 groups.

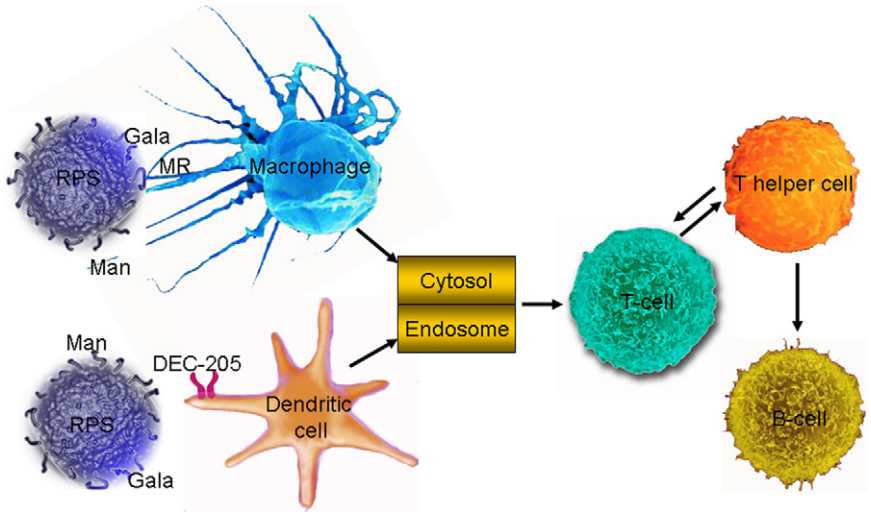

Fig. 6. Schematic depiction of the possible interactions between heteropolysaccharides and lymphocytes.

TEM cellular images clearly indicated that treatment of RPSs could lead to apoptosis in S-180 cells similar to the treatment of DDP. These results were consistent with previous reports on the suppression of tumor growth by mushroom polysaccharides mediated by either enhancing the host's immune system or by inducing apoptosis in tumor cells (Ma, Wang, Zhang, Zhang, & Ding, 2010; Zhu et al., 2007).

3.6. Structure–bioactivity relationship

As mentioned above, the heteropolysaccharides including RPS1 and RPS5 were demonstrated to be potential candidates for cancer therapy. They could stimulate T cell proliferation and induce apoptosis in S-180 cells. RPS1 inhibited tumor growth preferentially by direct cytotoxic effect while RPS5 mainly through immunomodulation with little side effects to the organs of the animals. These are crucial prerequisite for any anticancer or immunotherapeutic agents for clinical human use. Based on our previous studies on the structure and conformation of these branched heteropolysaccharides (RPSs) (Huang & Zhang, 2009; Huang et al., 2009, 2010), some relations between the structure, conformation and the bioactivity of RPSs could be inferred in the present study. First of all, the structure of a α-(1 → 4)-D-glucan main chain which is common to all RPSs seemed to contribute to the bioactivity, and the high water solubility of RPSs is essential to facilitate their cellular adsorption (Huang & Zhang, 2009). By comparing the differences in the monosaccharide composition of the side chains of RPSs and their bioactivity (immunopotentiation), it was found that those (including RPS1 and RPS5) with more galactose and mannose but without xylose in their side chains (Huang et al., 2010) showed higher bioactivity than those (including RPS3 and RPS4) with more xylose in their side chains (Huang et al., 2010). This suggested that the former structural features was more essential to the bioactivity of RPSs than the latter one.

Other than the sugar composition of the side chains, the molecular mass of RPS5 was 10 times more than that of RPS1 while RPS1 had a water solubility of 10 g dry weight/100 g water which was 5 times more than that of RPS5 (Huang et al., 2009) and RPS5 was more compact in shape (exponents ν of $\langle s^2 \rangle_z^{1/2} = kMw^{\nu}$ was 0.31 for RPS5and 0.43 for RPS1)(Huang et al., 2010). The physico-chemical properties of RSP1 including a smaller molecular mass and better water solubility could facilitate its absorption by cells and thus might explain its direct cytotoxicity towards S-180 cells. It has been reported that the water-soluble Lentinan with helical conformation plays a significant role in its recognition by immune cells (Zhang et al., 2007). RPS5 had a more compact size than RSP1 and is capable of having a nano-spherical chain conformation in water which might be another factor for enhancing its bioactivity. Despite the difference in geometry between the spherical shape for RPS and helical chain for Lentinan, they both have immunomodulatory activity, suggesting that the mechanism of the bioactivity in RPS might be different from that of Lentinan. The compact spherical structures and low viscosity of RPS in water could facilitate flow of fluid, which facilitate cellular contact and binding (Gu et al., 1998). Moreover, the spherical shape of the RPS with its localized hydroxide end groups could have stronger interaction through hydrogen bonding to the immune cells than linear polysaccharides. It has been reported that most of the mammalian cells express carbohydrate receptor(s) or carbohydrate transferase(s) on their cell surface. For example, the murine macrophage mannose receptor (MR) (Harris, Super, Rits, Chang, & Ezekowitz, 1992) and a homologous receptor, DEC-205 (Jiang et al., 1995), are expressed on macrophages or dendritic cells. Both receptors possess carbohydrate recognition domains, which are thought to increase both the affinity and diversity of carbohydrates bound to these receptors. In addition, liver parenchymal cells exclusively express high levels of receptors that efficiently bind to galactose (Park et al., 2001) and this might be the reason why RPS1 and RPS5 could protect the animal's liver. Immune cells with carbohydrate receptors are able to uptake heteropolysaccharide particles by phagocytosis. Thus, these particles could adhere to the cell surface as well as to be engulfed by plasma membrane. The nanosphere of heteropolysaccharide might be more efficiently taken up by the receptors such as MR or DEC-205 compared with homoglycans. They could be thought to be delivered to the endosomal compartment or the cytosol from which they are eventually presented to T cells (Varki et al., 2009). As a result, a T helper cell response would be induced, which might stimulate T cells and/or B cells, in accordance with the increase in CD4 and CD8 expression on T cells found in our *in vivo* data. A schematic depiction of the possible interactions of RPS to lymphocytes is shown in Fig. 6. The spherical RPS molecules could facilitate their binding with the receptors on the surface of macrophages or dendritic cells with their hydrophilic mannose or galactose side chains. These binding interactions could lead to the enhancement of the proliferation and differentiation of murine macrophages. Such immune reactions could induce stronger immune responses that inhibited the proliferation of tumor cells as previously reported (Varki et al., 2009).

4. Conclusions

The water-soluble branched heteropolysaccharides from RPJ (RPSs) could directly inhibit the proliferation of S-180 tumor cells via apoptosis and also enhance the immune responses and protect the vital internal organs of BALB/c mice. Such bioactivity might be due to the unique structural features of RPSs including the α-(1 → 4)-D-glucan main chain, water-soluble galactose and mannose side chains, as well as nano-spherical chain conformation which could be recognized easily by the surface receptors in the immune cells. Further studies on the bioactivity of these heteropolysaccharides that have such unique structural characteristics in human cancer cells are required in order for them to be developed into cancer therapeutic agents.

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