Asia PACIFIC BIOTECH NEWS

Antitumor Effects of a Refined Polysaccharide Peptide Fraction Isolated from *Coriolus versicolor*: *In vitro* and *in vivo* Studies

Ying Dong, Chiu-Yin Kwan, Zhi-Nan Chen and Mabel Mel-Po Yang

Department of Physiology, Faculty of Medicine,
The University of Hong Kong
5 Sassoon Road, Pokfulam, Hong Kong

Abstract

RPSP, a refined polysaccharide peptide fraction isolated by fast performance liquid chromatography (FPLC) from the crude powder of total peptide-bound polysaccharides of cultivated *Coriolus versicolor Cov-1* dose-dependently inhibited the proliferation of a human hepatoma cell line (HEPG2). The effective dose causing 50% inhibition following a 3-day exposure to RPSP was 243 ± 36 μg/ml for HEPG2. However, little or no inhibitory effects were detected in normal human fetal hepatocytes. On the other hand, in the pretreatment group, in which RPSP was administered i.p. for two weeks before sarcoma 180 inoculation in nude mice, the incidence of tumor growth was less (2 out of 5 mice) than that of the control group (all 5 mice). The tumor size of the control group was about 3–5 times bigger than that of the pretreatment group. In tumor-bearing nude mice, 5 days after sarcoma 180 inoculation, i.v. administration of RPSP significantly suppressed the growth of tumor mass. The inhibition rate was 93.6% on day 13. Furthermore, administration of RPSP did not cause any pathological lesions in vital organs of rabbits such as the heart, liver, spleen, lung and kidney. In conclusion, these results indicate that RPSP acts by directly suppressing the tumor cell growth *in vitro* and the prevention of *in vivo* growth of tumor mass is probably mediated also via its immunomodulating effects.

Introduction

Medicinal fungus mushrooms have been commonly used in the Orient as health food for hundreds of years. During the last decade some strains of mushrooms have received considerable attention for their possible antitumor effects (Mizuno *et al.*, 1990; Ma *et al.*, 1991; You *et al.*, 1994). One of the strains with very potent antitumor effects is *Coriolus versicolor Cov-1*. A group of polysaccharide peptides (PSP), isolated from the mycelium of deep layer cultivation of *Coriolus versicolor Cov-1* was found to possess remarkable antitumor and immuno-stimulant effects (Yang *et al.*, 1992; Li *et al.*, 1990). Similar finding, has also been reported for polysaccharide peptides, PSK, isolated from *Coriolus versicolor Krestin* (Tsukacoshi *et al.*, 1984; Fujii *et al.*, 1987; Kamisato and Nowakowski, 1988). Therefore, these mushroom-derived polysaccharide peptides, like many antitumor Chinese medicine preparations exert their activities primarily via immunomodulation (Hasegawa *et al.*, 1990; Mizoguchi *et al.*, 1986a, 1986b, 1989; Yano *et al.*, 1994). Recently, a peptide fraction with antitumor effects, RPSP, has been better refined by FPLC from the PSP powder extracted from the mycelium of *Coriolus versicolor Cov-1* (Yang *et al.*, 1992). In the present study, we further characterize the *in vitro* antitumor effects of RPSP on the human liver cancer cell line, HEPG2 and study its *in vivo* effects on the growth of tumor mass in mice inoculated with Sarcoma 180 cells. We observed that RPSP suppressed the proliferation of HEPG2, but not that of normal human fetal liver cells, QZG. We also demonstrated that RPSP significantly suppressed the *in vivo* growth of tumor mass probably via stimulating the immune system.

Materials and Methods

In vitro experiments

Culture medium and RPSP

RPMI medium 1640 (GIBCO, New York, USA) supplemented with 10% fetal bovine serum (GIBCO, New York, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (GIBCO BRL, New York, USA) was used for cell culture as the basal medium. RPSP was isolated by FPLC using DEAE-40 HR column and

the major peptide eluent was collected, desalted, concentrated and freeze-dried for use. From SDS-PAGE and electrofocusing electrophoresis, the molecular weight of RPSP was found to be about 11–12 kD and PI value was from 4.0–4.5 (Yang *et al.*, 1992). RPSP powder was dissolved in basal medium to the concentration of 10 mg/mL and was further diluted with the basal medium to the desired concentrations of between 20–2000 μg/mL.

Cellular proliferation studies

HEPG2 and the normal human fetal liver cell line (QZG) were originally purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. The seeded cells were cultured for 1 day in the basal medium. On the next day, the culture medium was replaced with medium containing desired concentrations of RPSP or the control medium. QZG and HEPG2 cells were seeded on 24-well plates at a density of 1.0 x 10^5 cells/well and were cultured for 3 days in medium with or without RPSP. The number of viable cells was identified and counted using the trypan blue dye exclusion test on day 3 of culture, and the cell numbers of HEPG2 were compared with those of QZG cells under the same experimental conditions.

In vivo experiments

Nude mice and Balb/c mice weighing 10–22 g, were kept in an air-conditioned room at 20–22^{0}C and fed with tap water and a commercial stock diet (PMI Feed Inc., Logan, USA) in the university animal facility following the guidelines for the use of laboratory animals in research and teaching. Tumor cells were obtained from standard stock culture (Cell Bank, Chinese Academy of Sciences, Shanghai) and used as inocula for *in vivo* growth study. Serum IgG and IgA were measured by using a modified radial immunodiffusion method (Fahey and Mckelvey, 1965). In the pretreatment groups, RPSP (0.5–2.0 mg, i.p.) was given daily for two weeks before inoculation of sarcoma 180 in nude mice. In the treatment groups, RPSP (2 mg, i.v.) was given every 48 hrs.

Histopathological examination

New Zealand rabbits of either sex, weighing 3–4 kg were kept and raised in the animal unit of the University of Hong Kong under standard laboratory conditions. Before histopathological examination, RPSP was administered intramuscularly 10 mg per day for one month. Pathological lesions were examined in vital organs including the heart, liver, spleen, lung and kidney by hematoxylin and eosin stain method (Disbrey and Rack, 1970).

Morphological methods

HEPG2 and QZG cells in RPSP-treated culture were examined under light microscope on day 3. These cells were then pelleted at 150 x g, resuspended in a drop of fetal bovine serum, smeared on a slide, air dried, stained with hematoxylin and eosin, and then observed for morphological changes at x1000 magnification (Yano *et al.*, 1994; Wylle *et al.*, 1984).

Results

Effects of RPSP on cell proliferation

Figure 1 shows that RPSP elicited dose-dependent inhibitor effects on the proliferation of HEPG2 cells. ED_{50} values (day 3) were 243 ± 36 μg/mL for HEPG2. In cultures of normal QZG, the proliferation of cells was slightly but significantly suppressed at concentrations > 1 mg/mL.

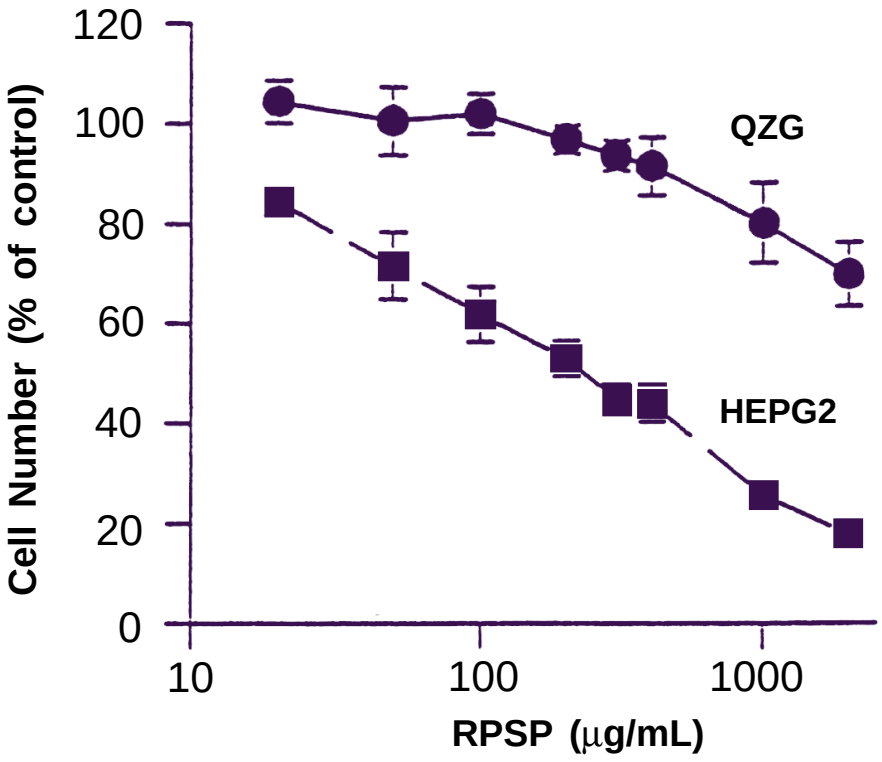

Figure 1. Cell growth following 72 hrs incubation with various concentrations of RPSP. Values are shown as mean ± SE of 3 separate experiments with duplicate wells. Note the marked inhibition of cell growth elicited as reduced cell number compared to the control.

Effects of RPSP on Morphological Changes

In HEPG2 cell culture, these cells showed normal appearance without any identifiable characteristics of apoptosis, such as cell shrinkage, chromatin condensation and nuclear fragmentation on day 3 indicating the lack of apoptosis, which was also supported by the lack of evidence of DNA fragmentation in tumor cells treated with RPSP (not shown).

In vivo experiments

Pretreatment of RPSP (5 weeks after injection of RPSP) in nude mice showed that the incidence of tumor mass (induced by sarcoma 180 cells implanted 2 weeks after RPSP injection was initiated) was significantly less (in 2 out of 5 mice) than that of the control group (saline injection, in all 5 mice). The tumor size of the control group was about 3–5 times bigger than that of the pretreatment group (Fig. 2).

After administration of RPSP in nude mice (1 mg/day x 15 days), the serum IgG levels increased from 2964 mg/L to 884 mg/L and the serum IgA levels increased from 489 mg/L to 1079 mg/L (Table 1). White blood cell counts also increased from 7914/mm^3 to 15 393/mm^3 (Table 2).

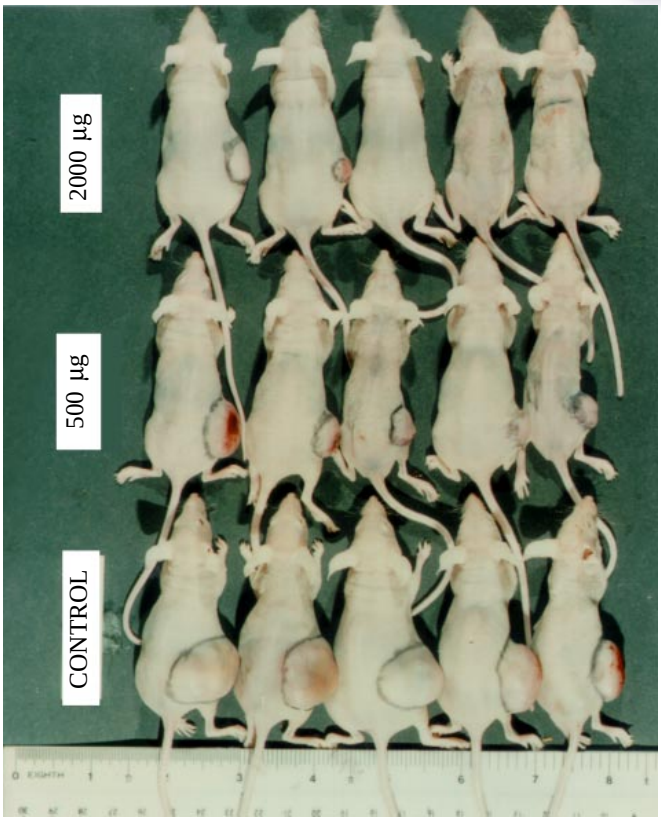

Figure 2. Mice were treated with daily i.p. injection of RPSP for 5 weeks. Sarcoma 180 cells were inoculated two weeks after starting the treatment of RPSP (0.5 mg and 2.0 mg). Note that the incidence of tumor mass was markedly lower in RPSP-treated mice.

Table 1. Effect of RPSP on serum IgG and IgA levels of nude mice.

Weeks		0	2
IgG mg/L (n=8)	Saline	2180.0 ± 186.7	2082.0 ± 164.7
	RPSP	2964.3 ± 139.4	6884.3 ± 552.8*
IgA mg/L (n=5)	Saline	459.0 ± 30.0	582.0 ± 59.9
	RPSP	489.5 ± 29.2	1079.8 ± 111.2*

*RPSP was given 1 mg (i.p.) x 15 days, $p < 0.01$, mean ± S.E.

Table 2. Effect of RPSP on WBC and neutrophil % of nude mice.

Weeks		0	2
WBC	Saline	9280.0 ± 554.0	8630.1 ± 625.4
	RPSP	7914.3 ± 715.1	15 392.9 ± 769.5*
Neutrophil %	Saline	5.8 ± 0.6	4.2 ± 0.8
	RPSP	3.7 ± 0.7	13.9 ± 2.1*

*RPSP was given 1 mg (i.p.) x 15 days, $p < 0.01$, $n = 8$, mean ± S.E.

In the treatment group, it was found that RPSP prolonged survival time and survival rate in Balb/c mice (Dong *et al.*, 1995). Furthermore, results in Table 3 shows that 5 days after sarcoma 180 cells inoculation, administration of RPSP (2 mg/day i.v., every 48 hours) progressively suppressed the growth of tumor mass during the treatment.

Pathological examination

Intramuscular administration of RPSP (10 mg/day, in rabbits for one month) did not cause any visible pathological lesions in vital organs including the heart, liver, spleen, lung and kidney (not shown).

Table 3. Effect of RPSP on the tumor size (X), growth rate (GR) and inhibition rate (IR).

	X ± SE (mm)		GR (%)		IR (%)
Days	Control	RPSP	Control	RPSP	
3	2.5 ± 0.2	2.9 ± 0.3	0	0	0
5	4.1 ± 0.4	4.5 ± 0.4	65.0	56.5	13.1
7	6.3 ± 0.6	5.6 ± 0.6	51.5	25.0	51.5
9	8.9 ± 0.7	6.0 ± 0.7	42.0	6.7	84.0
11	10.8 ± 0.7	6.2 ± 0.8	21.1	3.2	84.4
13	12.4 ± 0.8	6.3 ± 0.8	15.1	1.0	93.6

The data obtained were compared with those of control group. Five days after Sarcoma 180 cell implantation, nude mice were injected with RPSP (2 mg/day, i.v., every 48 hours). n=8. Growth rate represents the value (X_2–X_1) divided by X_1. Inhibition rate represents the value (growth rate of control – growth rate of RPSP) divided by growth rate of control. Note that RPSP progressively suppressed the growth of tumor mass after the treatment.

Discussion

In the present study, using better purified active constituent of the polysaccharide peptide powder of the *Coriolus versicolor Cov-1* (RPSP), we were able to demonstrate that RPSP, at concentrations < 1 mg/mL, directly inhibits the proliferation of HEPG2 cells without significant effects on the corresponding normal QZG cells. Therefore, RPSP produces selective *in vitro* inhibitory effects on the proliferation of tumor cell lines. This finding is consistent with previous observations on the antitumor effects of PSP isolated from *Coriolus versicolor Cov-1* (Li *et al.*, 1990; Yang *et al.*, 1992) and PSK isolated from *Coriolus versicolor Krestin* (Kamisato and Nowakowski, 1988; Fujii *et al.*, 1987). The main components of PSP and PSK in clinical application are molecularly distinct polysaccharide peptides (Yang and Yuan, 1986; Tsukagoshi *et al.*, 1984) and the antitumor effect of PSP was reported to be more potent than that of PSK (Li and Xu, 1987).

Apoptosis, a physiological process of programmed cell death has been widely studied as a pathophysiological event associated with the action of antitumor substances (Yano *et al.*, 1994; Sen and D' Incalci, 1992). It seems unlikely that RPSP inhibits the proliferation of tumor cell lines by inducing apoptosis since the normal morphological features and the lack of DNA fragmentation pattern of HEPG2 cells following treatment of RPSP suggest that RPSP does not act by inducing apoptosis in tumor cell lines.

In vivo studies show that administration of RPSP prior to tumor implantation significantly reduced the incidence of tumor growth (in 2 out of 5 mice) compared with that of control group (in all 5 mice), that IgG, IgA and WBC levels were all elevated compared with those of the control group on day 15 before tumor cell inoculation, and that RPSP treatment following the induction of tumor significantly suppressed the growth of tumor (the inhibition rate was 93% on day 13). These results collectively suggest that RPSP possibly acts by suppressing the growth of tumor via the stimulation of the immune system. This aspect of action of RPSP is also consistent with the immunopotentiating actions of PSP (Liu, 1989; Shiu *et al.*, 1992) and PSK (Kikuchi *et al.*, 1988; Niimoto *et al.*, 1988). Since RPSP did not cause any lesions on normal organs including the heart, liver, lung, kidney and spleen, as also supported by results from the lack of morphological changes in the normal QZG in the *in vitro* experiments, RPSP may be potentially an effective preventive antitumor agent possessing immunomodulating actions.

Acknowledgments

This work is supported by a grant from Winsor Health Products, Hong Kong. We wish to thank Mr. John S. L. Kwok, Mr. W. B. Wong, Dr. P. Zhu and Dr. W. H. Zhu for their assistance and advice.

References

Disbrey, B. D. and Rack, I. H. (1970). Histological laboratory methods. *Livingstone*, p. 50.

Dong, Y., Yang, M. M. P, Mi, L. and Kwok, J.S.L. (1995). The aniitumor effects of PCV *in vitro* and *in vivo. Int. J. Acupuncture and Oriental Med.* **6**, 1.

Fahey, J. L., and Mckelvey, E. M. (1965). Quantitative determination of serum immunoglobulins in antibody agar plates. *J. Immunol.* **94**, 84.

Fujii, T., Kano, T., Saito, K., Kobayashi, Y., Iijima, H., Matsumoto, T., Yoshikumi, C. and Taguchi, T. (1987). Effect of PSK on prohibited immunity of splenectomized mice. *Anticancer Res.* **7**, 845.

Hasegawa, I., Mizoguchi, Y., Tsutsui, H., Ichikawa., Y., Kawada, N., Morisawa, S. and Yamamoto, S. (1990). Effects of sho-saiko-to on interleukin 6 synthesis from mouse spleen cells. *J. Med. Pharm. Soc.* **7**, 24.

Kamisato, J. K. and Nowakowski, M. (1988). Morphological and biochemical alterations of macrophages produced by a glycan, PSK. *Immunopharm.* **16**, 89.

Kikuchi, Y., Kizzawa, I., Oomori, K., Kita, Y. and Kato, K. (1988). Effects of PSK on interleukin II production by peripheral lymphocytes of patients with advanced ovarian carcinoma during chemotherapy. *Jpn. J. Cancer Res.* **79**, 125.

Li, X. M. and Xu, L. Z. (1987). A study of anticancer effects of PSP and PSK on human tumor cell lines *in vitro. Acta Acad. Med. Shanghai* **14**, 23.

Li, X. Y., Wang, J. F., Zhu, P. P., Yang, S. X., Liu, L. and Ge, J. B. (1990). Immunodulating actions of PSP. *Eur. J. Pharmacol.* **183**, 904.

Liu, T. F. (1989). Clinical experience in the use of PSP in 41 cases of esophageal carcinoma treated by PSP and radiation. In: *Recent Advance in Cancer*, p. 63 (Chinese University Press, Hong Kong).

Ma, Y., Mizuno, T. and Ito, H. (1991). Antitumor activity of some polysaccharides isolated from a Chinese mushroom, "Huangmo", the fruiting body of *Hohenbuehelia serotina*. *Agric. Biol. Chem.* **55**, 2701.

Mizoguchi, Y., Sakagami, Y., Miyazima, K. and Yamamoto, Y. (1986a). The effects of Xiao-chai-hu-tang on lymphokine-activated killer (LAK) cell activity. *Allergy.* **35**, 1119.

Mizoguchi, Y., Fujinobu, Y., Kobayashi, K., Yamamoto, S. and Morisawa, S. (1986b). The effects of Xiao-chai-hu-tang on natural killer (NK) cell activity. *J. Med. Pharm. Soc.* **3**, 184.

Mizoguchi, Y., Kawada, N., Ichikawa, Y., Tanabe, I., Mizuno, M., Tomekawa, K., Hasegawa, I., Morisawa, S. and Yamamoto, S. (1989). Effect of sho-saiko-to on interleukin 1 production by hepatic sinusoidal endothelial cells. *J. Med. Pharm. Soc.* **6**, 172.

Mizuno, T., Inagaki, R., Kanao, T., Hagiwara, T., Nakamura, T., Ito, H., Shimura, K., Sumiya, T. and Asakura, A. (1990). Antitumor activity and some properties of water-insoluble heteroglycans from "Himematsutake", the fruiting body of *Agaricus blazei Murill. Agric. Biol. Chem.* **54**, 2897.

Niimoto, M., Hattori, T., Tamada, R., Sugimachi, T., Inobuchi, K. and Ogawa, N. (1988). Post-operative adjuvant immunochemotherapy with mitomycin C, futraful and PSK for gastric cancer. *Jpn. J. Surg.* **18**, 681.

Sen, S. and D' Incalci, M. (1992). Apoptosis: biochemical events and relevance to cancer chemotherapy. *FEBS Lett.* **307**, 121.

Tsukagoshi, S., Hashimoto, Y., Fujii, G., Kobayashi, H., Nomoto, K. and Orita, K. (1984). Krestin (PSK). *Cancer Treat. Rev.* **11**, 131.

Wyllie, A. H., Morris, R. G., Smith A. L. and Dunlop, D. (1984). Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* **142**, 67.

Yang, M. M. P., Chen, Z. and Kwok J. S. L. (1992). The antitumor effect of a small polypeptide from *Coriolus versicolor. Amer. J. Chin. Med.* **20**, 221.

Yang, Q. Y., Jong, S. C., Li, X. Y., Zhou, J. X., Chen, R. T. and Xu, L. Z. (1992). Antitumor and immunomodulating activities of the polysaccharide-peptide (PSP) of *Coriolus versicolor*. *Eos. Riv. Immunol. Immunofarm.* **12**, 29.

Yang, Q. Y. and Yuan, P. (1986). Isolation of the polysaccharide components of PSP. *J. Shanghai Teach. Univ.* **4**, 36.

Yano, H., Mizoguchi, A., Fukuda, K., Haramaki, M., Ogasawara, S., Momosaki, S. and Kojiro, M. (1994). The herbal medicine sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G_0/G_1, phase. *Cancer Res.* **54**, 448.

You, J. S., Hau, D. M., Chen, K. T. and Huang, H. F. (1994). Combined effects of chuling (*Polyporus umbellacus*) extract and Mitomycin C on experimental liver cancer. *Amer. J. Chin. Med.* **22**, 19.