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A Further Study on the Inhibition of Tumor Growth and Metastasis by Red Ginseng Acidic Polysaccharide (RGAP)

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Abstract – We have recently reported that red ginseng acidic polysaccharide (RGAP), isolated from Korean red ginseng (*Panax ginseng* C. A. Meyer), showed immunomodulatory antitumor activities, mainly mediated by nitric oxide (NO) production by macrophage. In this study, we examined the effect of RGAP on anticancer activity using lung carcinoma 3LL, sarcoma 180 and adenocarcinoma JC tumor cells transplanted into mice as well as antimetastatic activity using B16-F10 melanoma. When RGAP (300 mg/kg) were treated to mice implanted with one of the three kinds of tumor cells, the tumor weight significantly decreased compared with control mice. Tumor inhibition ratios of RGAP (300 mg/kg) in mice transplanted with lung carcinoma 3LL, sarcoma 180 and adenocarcinoma JC cells were 26.8%, 29.3% and 31.6%, respectively. Hundred mg/kg of RGAP did not cause a significant decrease in tumor weight compared with control group. When RGAP was administered i.p. with the dose of 100 and 300 mg/kg in B16-F10 melanoma-bearing mice, lung metastasis were reduced significantly in mice. Corrected phagocytic index was also remarkably increased by RGAP. These results suggest that stimulation of phagocytic activity of macrophages may be a mechanism for *in vivo* anticancer and antimetastasis activities of RGAP.

Keywords – *Panax ginseng;* Araliaceae; tumor cells; Red Ginseng Acidic Polysaccharide (RGAP); anticancer activity; lung metastasis.

Introduction

It has been widely accepted that cancer therapy using biological response modifiers aims at controlling tumor growth by the augmentation of the host defense immune systems (*Chihara et al.*, 1987). Metastasis is one of the major causes of mortality in cancer. During the metastatic cascade, metastasizing tumor cells interact with various host cells, extracellular matrices and basement membrane components (Albelda, 993; Murph and Docherty, 1993; Yano *et al.*, 1994). Such adhesive interaction may enhance the survival or invasiveness of the tumor cells (Julian and Varner, 1993; Kohn and Liotta, 1995). Therefore, regulation of tumor cell metastasis may help in the development of antimetastatic therapies.

We have previously reported that red ginseng acidic polysaccharide (RGAP), isolated from Korean red ginseng (*Panax ginseng*), has been found to induce the proliferation of spleen cell, and decrease antibody forming cell response to sheep red blood cells and stimulate nitric oxide production of murine macrophages (Park *et al.*, 2000; Park *et al.*, 2001). RGAP has also recently been found to exhibit immunomodulating anticancer activity in a murine transplanted with tumor cells (Kim *et al.*, 2002).

The present report discusses the examination of the inhibitory effect of RGAP on the growth of sarcoma-180, 3LL lung carcinoma and JC mammary adenocarcinoma transplanted subcutaneously into mice and on the lung metastasis produced by B16-F10 melanoma.

Materials and methods

Reagents – Korean red ginseng, made by steaming and drying fresh ginseng root KTG-303-003 (*Panax ginseng* C.A. Meyer), was kindly given from Korea Ginseng Corporation (Daejeon, Korea). Bovine serum albumin (BSA), 2-Mercaptoethanol (2-ME), 3-(4,5-Dimethyl-thia zol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), protease and dialysis membranes were purchased from Sigma Chemical Co. (St. Louis, USA). Absolute alcohol and sulfuric acid were purchased from Fluka (Buchs, Switz

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erland). PBS (pH 7.4), Earle's balanced salt solution (EBSS), HEPES, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycine solution, DMEM and RPMI 1640 medium were purchased from GIBCO (Grand Island, USA). All other chemicals and reagents used were of the best grade available.

Preparation of RGAP (Red Ginseng Acidic Polysaccharide) – RGAP was obtained from Korean red ginseng as described previously (Park *et al.*, 2000). Briefly, Korean red ginseng powder was percolated with 5 volumes of 85% ethanol to extract off ethanol-soluble materials. Remaining residues were then percolated with 5 volumes of distilled water and the resulting water-soluble fractions were concentrated with a vacuum evaporator. The concentrates was dialyzed against water to completely remove small molecules less than 10 KDa to obtain non-dialyzable fraction consisting of acidic polysaccharide (yield : about 5%). RGAP was confirmed to be the acidic polysaccharide fraction composed of about 56.9% uronic acid, 28.3% neutral sugar and less than 1% protein. The analysis of component sugars in RGAP by GC revealed that the polysaccharides in RGAP contained about 51.8 mole % glucuronic acid, 26.1 mole % glucose and 5.1 mole % galacturonic acid as major components, and arabinose, rhamanose, and glactose as minor components (Kim *et al.*, 2002). Less than 0.006EU (endotoxin unit) of endotoxin was present in 1 mg of RGAP as tested by *Limulus amebocyte* lysate assay (Sigma, USA). This level of endotoxin did not affect the experimental result obtained by RGAP. RGAP was supplied in a solution of PBS and maintained at -20°C Before use, RGAP was diluted in PBS and the appropriate concent rations in 100 μl volume were administrated to mice.

Cell culture – 3LL lung carcinoma, B16-F10 melanoma, sarcoma 180 and JC mammary adenocarcinoma cells obtained from the ATCC. 3LL and B16-F10 cells were maintained for 3-4 days passages and growth in a monolayer with DMEM supplemented with 10% FBS, 4 mM glutamine, 1.5 g/L sodium bicarbonate, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2/humidified air. Sarcoma-180 and adenocarcinoma JC cells were routinely grown in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 units/ml pen icillin and 100 μg/ml streptomycin.

Mice – Specific pathogen-free female C57BL/6, BA LB/c, BDF1 and ICR mice were purchased from Daehan Laboratory Animal Research Center Co. (Eumsong, Ko rea) and acclimated for at least 1 week. All animals were maintained on gamma-irradiated Jeil Lab Chow (Daejon, Korea) and ultraviolet-irradiated tap water *ad libitum*. Mice at 5-6 weeks of age were used as the experimental animals. The animal quarters were strictly maintained at 22 ± 2°C and 55 ± 5% relative humidity. A twelve-hour light/dark cycle was used with an intensity of 150-300 lux.

Solid tumor inhibition assay - Inhibition of lung carc inoma 3LL growth transplanted into the mouse : BDF1 mice were subcutaneously implanted on the back with 0.2 ml of tumor cells (1×10^5) in PBS. The mice were randomly assigned to several experimental groups at one day after the implantation. The mice inoculated were divided into control group and RGAP treatment group. One hundred or 300 mg/kg of RGAP was intraperitoneally (i.p.) treated daily for seven consecutive days. On day 20, the mice were sacrificed and tumor weights were measured. Tumor inhibition ratio (%) was calculated as follows: [1-average tumor weight of treated group/average tumor weight of control group]×100

Inhibition of sarcoma 180 growth transplanted into the mouse : Under sterile condition, 5×10^5 of tumor cells was subcutaneously inoculated to ICR mice in 0.2 ml/mouse. The mice were randomly divided into control and RGAP treated groups (100 or 300 mg/kg) at one day after the implantation. On day 22, the mice were sacrificed and tumor weights were measured.

Inhibition of adenocarcinoma JC growth transplanted into the mouse : BALB/c mice were subcutaneously implanted on the back with 0.2 ml of tumor cells (2×10^5) in PBS. The mice were randomly assigned to control and RGAP treated groups (100 or 300 mg/kg) at one day after the implantation. On day 30, the mice were sacrificed and tumor weights were measured.

Lung metastasis assay – The lung metastasis was ass essed by means of B16-F10 cells, a metastatic subline of murine B16 melanoma, injection into the lateral tail vein of mice. Five C57BL/6 mice per group were given an i.v. injection of B16-F10 melanoma (2×10^5). The treatment of RGAP was begun the next day after tumor inoculation. The mice were killed at 15 days after tumor inoculation. The lung was fixed in Bouin's solution and the lung tumor colonies were counted under a dissecting microscope.

Phagocytic activity assay – Phagocytic function was determined by the method of Biozzi *et al.* (1954). One day after 7 consecutive RGAP administration once daily, a suspension of carbon particle (Rotring Drowing Ink, Germany) in 1% gelatin solution (1:5) was injected to ICR mice via tail vain (0.1 ml/10g body weight). A blood sample 20 μl was taken from the retro-orbital plexus with heparinized capillary at 2 and 10 min after carbon injection and transferred into 1 ml of 0.1% $NaCO_3$. The absorbance

at 600 nm was measured. The phagocytic index (K) and corrected phagocytic index (a) were calculated by means of the following equation.

$K = \text{Log } OD_{t_1} - \log OD_{t_2}/t_2\text{-}t_1$

$a = \sqrt[3]{K\ P_c/P_o}$

OD_1, OD_2 : the optical density at time t_1 and $time_2$, respectively

P_c : body weight

P_o : liver weight + spleen weight

Statistical analysis of data – Data obtained from the pharmacological experiments were expressed as mean± S.D. Differences between the control and test groups in this experiment were measured for statistical significance by Student's t-test or analysis of variance (ANOVA).

Results and Discussion

As reported earlier, the physical and chemical properties of RGAP have been well established (Kwak *et al.*, 2003). RGAP, showing B-cell specific mitogen activity, caused a stimulation of spleen cells proliferations *in vivo* and *in vitro* (Park *et al.*, 2001). We previously reported that RGAP strongly increased life span of sarcoma 180-bearing mice and decreased tumor weight of B16-tumor transplanted in mice (Kim *et al.*, 2002). In this study, for complete confirmation as to antitumor activity of RGAP we examined the effect of RGAP on the growth of sarcoma-180, 3LL lung carcinoma and JC mammary adenocarcinoma transplanted subcutaneously into mice and on the lung metastasis produced by B16-F10 melanoma.

The effect of RGAP on anticancer activity was evaluated using several tumor cells *in vivo*. An i.p. injection of 100 and 300 mg/kg RGAP was given to mice at the next day after subcutaneous inoculation of lung carcinoma 3LL, sarcoma 180 and adenocarcinoma JC tumor cells, respectively. After 20 to 30 days, the tumor-inhibitory effect of RGAP was evaluated by measuring tumor weight both of control group and RGAP-treated mice group. As shown in Table 1, 2, 3 when RGAP (300 mg/kg) was treated to mice implanted with one of the three kinds of tumor cells, the tumor weight was significantly decreased as compared with that of control mice ($p<0.01$). Tumor inhibition ratio of RGAP (300 mg/kg) on mouse lung carcinoma 3LL, sarcoma 180 and adenocarcinoma JC cells were 26.8%, 29.3% and 31.6%, respectively. However, an administration of 100 mg/kg of

Table 1. Antitumor activity of RGAP in lung carcinoma 3LL -bearing mice

Group	Dose (mg/kg)	No of mice	Tumor weight (g,mean±S.D.)	Inhibition ratio (%)	Significance (p<)
Control	-	10	2.24±0.47	-	-
RGAP	100	7	2.14±0.49	4.5%	n.s.[a)]
	300	7	1.64±0.38	26.8%	0.01

DBF1 mice were subcutaneously implanted on the back with 0.2 ml of tumor cells (1×10^5) in PBS. RGAP was i.p. administered at 24 h after inoculation as PBS solution for seven consecutive days. On day 20, the mice were sacrificed and tumor weights were measured.
[a)] Not significance

Table 2. Antitumor activity of RGAP in sarcoma 180 -bearing mice

Group	Dose (mg/kg)	No of mice	Tumor weight (g,mean±S.D.)	Inhibition ratio (%)	Significance (p<)
Control	-	8	1.47±0.10	-	-
RGAP	100	7	1.42±0.35	3.4%	n.s.[a)]
	300	7	1.04±0.18	29.3%	0.01

ICR mice were subcutaneously implanted on the back with 0.2 ml of tumor cells (5×10^5) in PBS. RGAP was i.p. administered at 24 h after inoculation as PBS solution for seven consecutive days. On day 22, the mice were sacrificed and tumor weights were measured.
[a)] Not significance

Table 3. Antitumor activity of RGAP in adenocarcinoma JC -bearing mice

Group	Dose(mg/kg)	No of mice	Tumor weight (g,mean±S.D.)	Inhibition ratio (%)	Significance (p<)
Control	-	11	1065±162	-	-
RGAP	100	8	968±175	9.1%	n.s.[a)]
	300	7	728±182	31.6%	0.05

BALB/c mice were subcutaneously implanted on the back with 0.2 ml of tumor cells (2×10^5) in PBS. RGAP was i.p. administered at 24 h after inoculation as PBS solution for seven consecutive days. On day 30, the mice were sacrificed and tumor weights were measured.
[a)] Not significance

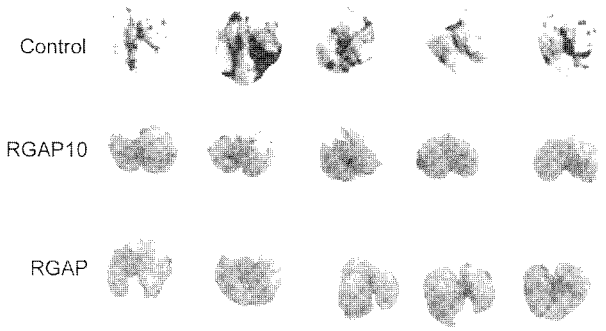

Fig. 1. Inhibition effect of RGAP on lung metastasis in B16-F10 melanoma tumor-bearing mice. Five C57BL/6 mice per group were inoculated i.v. with B16-F10 melanoma (2×10^5) and administered i.p. with RGAP for seven consecutive days after tumor inoculation. On day 15 after tumor inoculation, mice were killed and lung metastases were observed. RGAP 100 and 300 indicates 100 mg and 300 mg/kg of RGAP administration group, respectively.

Table 4. Effect of RGAP on carbon clearance activity in ICR mice

Group	Dose (mg/kg)	Corrected phagocytic index (a)	Relative phagocytosis (%)
Control	-	3.711±0.914	100
RGAP	100	5.456±0.956	147
	300	6.028±0.887	162
	300	5.553±0.827	150

RGAP was i.p. administered daily for 7 consecutive day. After 24 h of last treatment carbon clearance was determined as described in material and methods. Each value represents mean±S.D. of 4 mice. Significant difference from the control group; * $p<0.01$.

RGAP could not cause a significant decrease in tumor weight compared with that of control group.

Tumor metastasis is one of the important event in the process of cancer (Fry *et al.*, 1996). We therefore, tried to investigate whether or not RGAP could inhibit lung metastasis produced by the i.v. inoculation of B16-F10 melanoma cells. As shown in Fig. 1, 300 mg/kg admi nistrations of RGAP significantly inhibited the lung metastasis as compared with the untreated control (PBS). The significance of their antimetastatic effects was recog nized even at a dose of 100 mg/kg, which did not show inhibitory effect on the growth of mouse transplantable tumor cells.

As reported earlier in our paper, RGAP was found to exhibit tumoricidal activities through the stimulation of macrophages (Kim *et al.*, 2002). The antitumor function of macrophages may comprise two mechanisms: cell to cell contact between effector cells and target cells, and discharge of factors like cytokines (TNF-α) and nitrogen intermediate (Hibbs *et al.*, 1977; Hamilton and Adams, 1987). Our previous studies have revealed that RGAP induced TNF-α and NO production of macrophages. To show light on the correlation between stimulated antitumor activity of macrophages and cell to cell contact mec hanisms, we have examined the effect of RGAP on reticuloendotherial system (RES) using BALB/c mice. The effect of RGAP on RES was assessed by comparing the clearance time of carbon particles from blood stream. As shown in Table 4, corrected phagocytic index (a) was remarkably increased by all dose of RGAP, and very low dose of RGAP (30 mg/kg) had a significant enhancement in phagocytic activity of macrophages.

Defective monocyte function has been associated with a variety of human malignancies. Recent studies demonstrated that patients with breast cancer have a significant decrease in the absolute number of monocytes and phagocytic activity (Kastelan *et al.*, 2004). We found that PGAP treatment at low dose initiated phagocytic response, suggesting that RGAP prevented metastasis of tumor melanoma in mice by stimulation of phagocytic activity of macrophages.

In conclusion, this study demonstrated that administration of RGAP inhibited growth of several tumor cells *in vivo* and prevented lung metastasis of B16-F10 melanoma in mice and that the antitumor action of RGAP was associated with the activation of non-specific phagoc ytosis as well as augmented TNF-α and NO production of macrophages.

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