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Damage of radioprotection and antitumor effects of water-soluble propolis

Kaoru Terai[1], Myung-Sun Ryu[1], Yuka Itokawa[1], Toshihiro Maenaka[1], Takashi Nakamura[1], Takeo Hasegawa[1], Insuk Choi[1], Torao Ishida[3,4] and Yeunhwa Gu[1,4,*]

[1]Graduate School of Health Science, Suzuka University of Medical Science, 1001-1 Kishioka-cho, Suzuka-shi, Mie 510-0293, Japan; [2]Department of Clinical Nutrition, Faculty of Health Science, Suzuka University of Medical Science, 1001-1 Kishioka-cho, Suzuka-shi, Mie 510-0293, Japan; [3]Department of Acupuncture Moxibustion, Faculty of Acupuncture Moxibustion, Suzuka University of Medical Science, 1001-1 Kishioka-cho, Suzuka-shi, Mie 510-0293, Japan; [4]Hi-tech Research Center, Suzuka University of Medical Science, 1001-1 Kishioka, Suzuka, Mie 510-0293, Japan

SUMMARY

Some natural products are able to inhibit radiation effects and exert an antitumor effect with fewer adverse reactions; however, their antitumor effects are less than those of widely-used synthetic drugs. Propolis is a natural material that has been attracting attention, and we extracted this material with water and investigated the effect of continuous propolis administration on radioactivity-induced reduction of hemocytes, in addition to the antioxidant and antitumor effects of propolis. Following a 1-week adjustment period, water-soluble propolis was administered intraperitoneally to male ICR mice at a dose of 100 mg/kg every other day for 2 weeks. Following administration, 2 Gy whole-body irradiation was performed and the counts of leukocytes, lymphocytes, and granulocytes and monocytes in the peripheral blood were determined 1, 3, 7, 15 and 30 days after irradiation. These cells were considered since they are closely associated with immunity to radioactivity. In a second experiment, water-soluble propolis was similarly administered to the mice for 2 weeks after a 1-week adjustment period, and 2 Gy whole-body irradiation was performed. The antioxidant effects in hemocytes were then investigated using 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), a radical generator. In a third experiment, 1×10^6 Sarcoma-180 cells were inoculated into the right thigh of mice, which were divided into four groups: control, water-soluble propolis-treated, 6 Gy irradiated and water-soluble propolis-treated + 6 Gy irradiated groups, and changes in tumor size were measured for 20 days. Statistical analysis was conducted using ANOVA for multiple groups. In the three experiments, administration of water-soluble propolis inhibited the reduction of hemocytes caused by whole-body irradiation, showed antioxidant effects against radioactivity, and inhibited tumor growth, respectively. In conclusion, our data suggest that the antioxidant effect of water-soluble propolis inhibits hemocyte reduction caused by whole-body irradiation and enhances immunological inhibition of tumor growth.

Key words: Antitumor effect; Propolis

*Correspondence: Yeunhwa Gu, Graduate School of Health Science, Suzuka University of Medical Science, 1001-1 Kishioka-cho, Suzuka-shi, Mie 510-0293, Japan. E-mail: gu@suzuka-u.ac.jp

INTRODUCTION

Propolis is a natural material collected by bees from the skin, sap, and bud and so on of the tree. It

is known that propolis has contained various elements, polyphenoles, flavonoids, phenolic acid and their esters, caffeic acid and their esters and phenolic aldehydes and ketones, respectively (Nada *et al.*, 2003). It has been used in folk medicine.

In present studies, water-soluble propolis (WSP) has been shown various pharmacological effects including anti-inflammatory (Dobrowolski *et al.*, 1991; Khayyal *et al.*, 1993; Strehl *et al.*, 1994; Mirzoeva *et al.*, 1996; Volpert *et al.*, 1996), antimicrobial (Dobrowolski *et al.*, 1991), antioxidant (Scheller *et al.*, 1989; Krol *et al.*, 1990; Sud'ina *et al.*, 1993; Pascual *et al.*, 1994), immunostimulatory, antihyperglycemic and antitumor activities (Grunberger *et al.*, 1988).

Some the influences on the radiation that uses the WSP (El-Ghazaly *et al.*, 1995) and the researches of the antitumor effect are performed. This time, we report that effects of blood cell and antioxidant for whole-body irradiation of mice and influence of Sarcoma-180 antitumor *in vivo* using WSP.

MATERIALS AND METHODS

Water-soluble propolis

Lumps of crude Brazilian propolis (500 g) were powdered and mixed with 2 l of water at 50°C for 2 hours (Suzuki *et al.*, 2002). Then the suspended material was separated by centrifugation at 4.5×10^4 rpm for 10 min. The resulting pellet was extracted with 2 l of water and the material again centrifuged. The combined supernatants were passed through filter paper and freeze-dried to produce WSP yield 56.8 g (11.3%). WSP was suspended in saline and administered to mice at a does of 100 mg/kg every other day. WSP administration continued until the end of the experiments.

Animals

Five-week-old mail ICR mice with a mean weight of 18 - 20 g were purchased from Japan SLC Inc. and kept under standard conditions (room temperature 22 ± 3°C, humidity 60%) with free access to food (CA-1, Japan Clare, Inc.) and drinking water. The mice were acclimated to the breeding and experimental environment for 1 week prior to the experiments.

Irradiation

X-ray irradiation was administered to each mouse using an X-ray generator designed for animal use (Phillips, Inc.). The table was rotated at a constant speed so that these mice were irradiated evenly. The conditions for irradiation were: source voltage, 200 kV; rate of radiation, 0.35 Gy/min; and supplemental filter, 0.1 mm Cu + 1 mm Al.

Measurement of peripheral blood cell count

Mice were divided in 4 groups, control, WSP, irradiation alone and combined with WSP and irradiation. After acclimation, control and irradiation alone group of mice were i.p. injected with saline, WSP and combined with WSP and irradiation group of mice were injected with WSP 2 weeks of every other day. Irradiation groups had 2 Gy whole body irradiation and 10 ml of peripheral blood was collected with capillary tube from tail vein then counted with an automated blood cell counter (Celltac-α MEK-6318, Nippon Koden Inc.). The numbers of peripheral leukocytes, lymphocytes, granulocytes and monocytes, which all have relatively high sensitivity to radiation and primary cells of the immune system, were counted. Measurements were done at 1 day before irradiation and at 1 day, 3 days, 7 days, 15 days and 30 days after irradiation.

Chemiluminescence measurement

After acclimation, mice were divided in 4 groups same as the experiment of blood cell count. WSP was injected to mice for 2 weeks. After 2 Gy whole-body irradiation, whole blood was collected from mouse hearts by puncture with 23-G needle under anesthesia, mixed with heparin, and either centrifuged (10 min at 1,200 rpm 2°C) to separate blood plasma. Blood plasma was suspended 1 : 100 in PBS to 200 μl then added 200 μl of AAPH (2,2'-azobis[2-

amidinopropane]). After warm 37°C for 120 sec, added 200 μl of luminal, chemiluminescence intensity was recorded using a luminescence reader (ALOKA BLR-201).

Measurement of WSP injection or combined with X-irradiation on tumor growth

After acclimation, mice were divided in 4 groups same as the experiment of blood cell count. WSP was injected to mice for 2 weeks then 1×10^6 Sarcoma-180 cells were injected into the right femoral region. When tumor grew to 10 mm in diameter, irradiation groups were treated with 3 times of 2 Gy irradiation every other day and all groups of tumor diameters were measured with a caliper for 20 days. Tumor volumes were calculated using the formula: $V = ab^2/2$, where "a" was the shortest tumor diameter and "b" was the longest tumor diameter measured.

Statistical analysis

Experimental values are given as mean ± standard error of the mean (SE). All experiments of statistical analysis were performed using a parametric ANOVA test among the groups to determine significant difference.

RESULTS

Figure 1 shows the changes in the number of hemocytes, (leukocytes, lymphocytes, monocytes and granulocytes) caused by irradiation. The leukocyte counts in the water-soluble propolis-treated and control groups were $195.16 \times 10^2 \pm 25.41 \times 10^2$ cell/μl and $125.73 \times 10^2 \pm 13.16 \times 10^2$ cell/μl , respectively (Fig. 1-a), showing a significant increased in leukocytes with propolis administration ($P < 0.01$). Following 2 Gy whole-body irradiation, the number of hemocytes rapidly decreased and reached a minimum 3 days after irradiation, before recovering gradually. The number of hemocytes in the water-soluble propolis-treated + irradiated group was generally higher than that in the irradiated group, with a significant difference between these groups on the day before irradiation, and after the minimum leukocyte count (day after irradiation: $P < 0.05$; 7, 15 and 30 days after irradiation: $P < 0.01$). Lymphocytes in the water-soluble propolis and control groups were $109.17 \times 10^2 \pm 17.67 \times 10^2$ cell/μl and $82.90 \times 10^2 \pm 11.40 \times 10^2$ cell/μl, respectively (Fig. 1b), showing that propolis administration increased the number of lymphocytes significantly ($P < 0.01$). Immediately after irradiation, the number of hemocytes rapidly decreased and all values in the irradiated group were statistically significantly different (3 days after irradiation: $P < 0.05$; 1, 7, 15 and 30 days after irradiation: $P < 0.01$). The number of lymphocytes in the irradiated and propolis-treated + irradiated groups 3 days after irradiation (minimum values) decreased from those on the day before irradiation by 8.9% and 21.2%, respectively. The monocyte counts of the water-soluble propolis-treated and control groups were $45.9 \times 10^2 \pm 7.7 \times 10^2$ cell/μl and $27.68 \times 10^2 \pm 4.57 \times 10^2$ cell/μl, respectively (Fig. 1c). Monocytes did not decrease immediately after irradiation, but rather on the day following irradiation, reached a minimum 3 days after irradiation, and then gradually recovered. Propolis administration increased the number of monocytes significantly ($P < 0.05$), showing statistically significant differences on the day after irradiation and 15 and 30 days after irradiation (day after irradiation: $P < 0.05$; 15 and 30 days after irradiation: $P < 0.01$). The number of monocytes in the irradiated and propolis-treated + irradiated groups 3 days after irradiation (minimum values) decreased from those the day before irradiation by 18.1% and 27.4%, respectively. The granulocytes also showed a tendency to increase with propolis administration (Fig. 1d). The number of granulocytes in the irradiated group reached a minimum 3 days after irradiation and recovered gradually thereafter; however, that of the propolis-treated + irradiated group reached a minimum the day after irradiation and recovered slightly earlier. No statistically significant difference in the number of granulocytes was found between the groups at any time point.

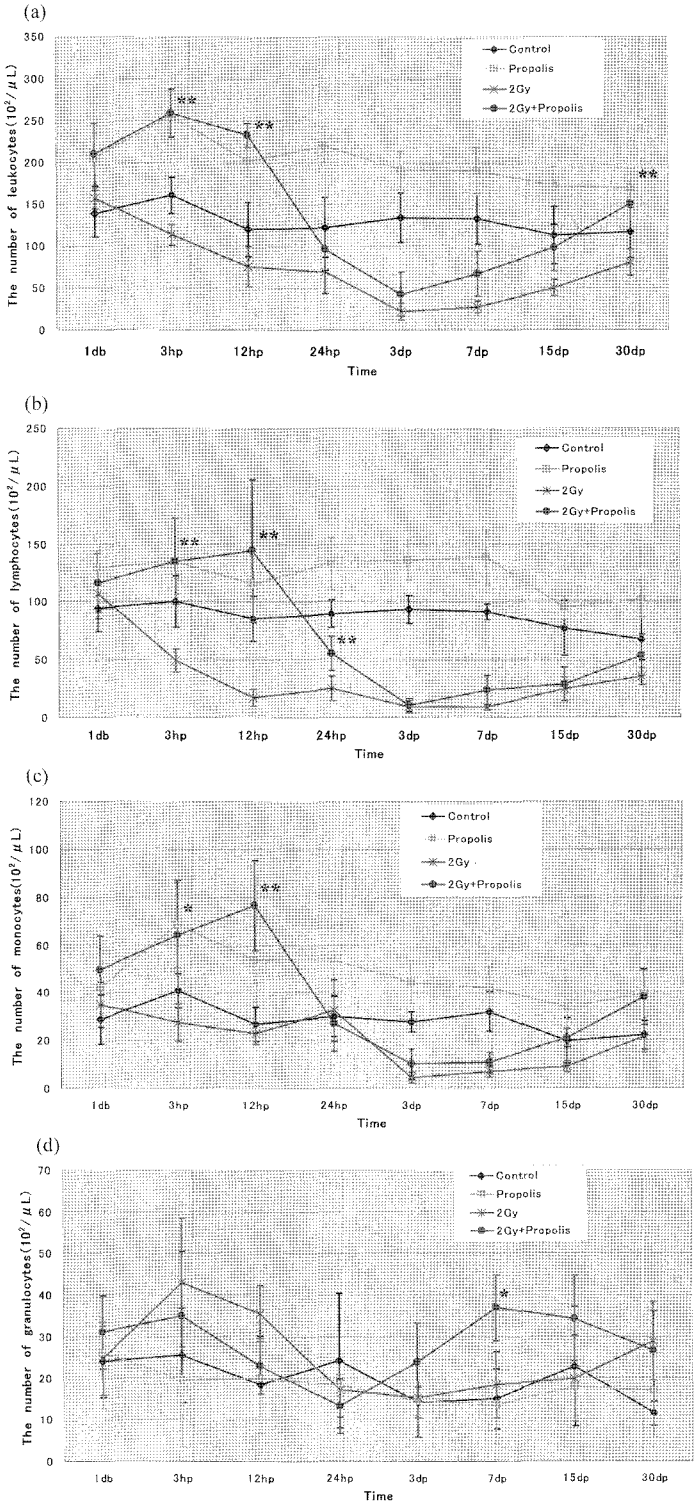

Fig. 1. The change in the number of cells in the blood taken from the tail vein of whole body irradiated mice. Each lineargram represents the mean value ± SE in (a) Leukocytes, (b) Lympocytes, (c) Monocytes and (e) Granulocytes from 10 mice (M). Significantly different $^{*}P < 0.05$, $^{**}P < 0.01$ Control vs WSP, 2Gy vs WSP+2Gy, respectively.

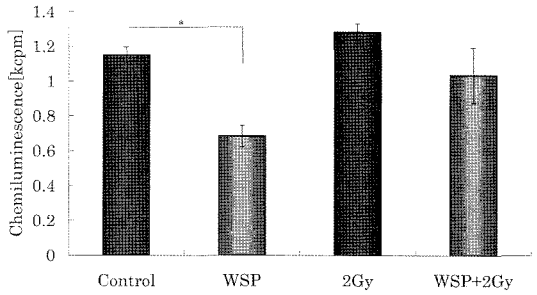

Fig. 2. Antioxidant activity of WSP in the blood plasma taken from the heart of mice. Each bar represents the mean value ± SE from 6 mice (M). Significantly different $^{*}P < 0.05$.

Figure 2 shows the antioxidant activity for the control, water-soluble propolis-treated, 2 Gy irradiated and water-soluble propolis-treated + 2 Gy irradiated groups, determined using the AAPH method. In the non-irradiated groups, statistically significant differences were found between the control and water-soluble propolis-treated groups ($P < 0.05$). In irradiated groups, no statistically significant differences were found between the groups, but the water-soluble propolis-treated + irradiated group showed a higher antioxidant activity, compared with the irradiated group.

Figure 3 shows the rate of tumor growth in each group for 20 days. Administration of water-soluble propolis delayed tumor growth. Irradiation also inhibited tumor growth, and administration of water-soluble propolis in addition to irradiation further delayed the tumor growth. However, no statistically significant differences were found among the groups at all determination points. The tumor growth rates (mean ± SE) 20 days after tumor implantation were 15.7 ± 2.8, 10.0 ± 2.7, 8.1 ± 1.4 and 6.5 ± 0.7 for the control, water-soluble propolis-treated, irradiated and water-soluble propolis-treated + Gy irradiated groups, respectively.

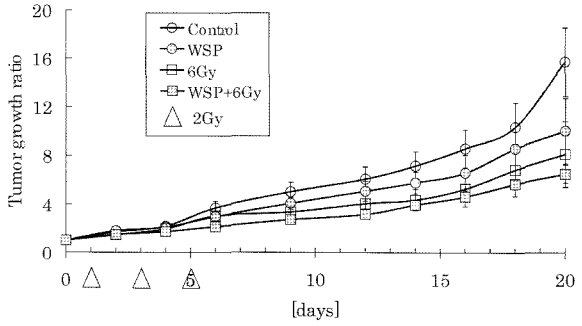

Fig. 3. The change of the tumor ratio of Sarcoma-180 at the right femoral region after various treatments. Each lineargram represents the mean value ± SE for 7 mice (M).

DISCUSSION

Whole-body irradiation is known to cause free radical-induced DNA damage and carcinogenesis, and to compromise the immune system due to its effect on leukocytes (Riley *et al.*, 1994). To evaluate the effects of water-soluble propolis on irradiation-induced damage, we investigated changes of hemocytes in the peripheral blood, inhibition of oxidation induced by AAPH, a radical generator, and changes in tumor growth of Sarcoma-180 following propolis administration. The effects of irradiation and water-soluble propolis on hemocytes (i.e., lymphocytes, monocytes, granulocytes and leukocytes), which are directly associated with the immune system, is shown with time in Figure 1. Administration of propolis prior to irradiation increased the hemocyte count significantly. Hemocytes were markedly reduced by irradiation, but propolis administration inhibited this reduction and increased the minimum level. Nada *et al.* have reported that single intraperitoneal administration of water-soluble propolis at a dose of 50 mg/kg significantly increases leukocytes 3 and 7 days after administration, with a concomitant increase in the weight of the spleen (Nada *et al.*, 2003), and also reported that leukocyte count and spleen weight increased after repeated oral administration of water-soluble propolis at a dose of 50 mg/kg 5, 10 and 15 days before determination (Orsolic *et al.*, 2003, 2004). Kimoto *et al.* showed both *in vivo* and *in vitro* inhibitory effects of artepillin C, which is a component of propolis, on the growth of various tumors, and

reported that cytotoxicity and adjuvanticity increased in lymphocytes (Kimoto *et al.*, 1998).

Hydroxyl radical (·OH) and superoxide radical ($O2^-$) are free radicals that are induced by irradiation. The OH radical is known to oxidize lipids to peroxy radicals (ROO·) and to increase lipid peroxide levels (Riley *et al.*, 1994). AAPH, a soluble azo compound, is a free radical initiator that generates radicals through simultaneous thermolysis, which results in oxygen inclusion into peroxy radicals (Olsher *et al.*, 2005). These radicals can then abstract a hydrogen atom from various molecules, combining with the hydrogen to stabilize themselves while inducing a radical chain reaction in the substances undergoing hydrogen loss. In this study, under conditions with and without irradiation, water-soluble propolis inhibited AAPH-induced peroxy radical formation (Fig. 2). Nagai *et al.* have reported the antioxidant and radical scavenging effects of water-soluble propolis on DPPH and hydroxyl radicals (Nagai *et al.*, 2004), and Cos *et al.* showed similar effects for a caffeic acid ester extracted from propolis (Cos *et al.*, 2002). El-Ghazaly *et al.* have reported that water-soluble propolis suppresses inflammation caused by whole-body irradiation (El-Ghazaly *et al.*, 1995), and also showed that superoxide dismutase (SOD) activity is increased in hemocytes following water-soluble propolis treatment of both irradiated and non-irradiated rats. SOD inhibits oxygen toxicity by catalytic scavenging of free radicals that would otherwise cause tissue damage (Fridovich *et al.*, 1983). Hence, propolis is a natural antioxidant that enhances SOD activity and inhibits production of oxides from lipid oxides (Amdam *et al.*, 2003; Sugimoto *et al.*, 2003; Celli *et al.*, 2004; Matsui *et al.*, 2004; Fuliang *et al.*, 2005), and Kobayashi *et al.* showed the O_2-scavenging effect of propolis at super critical state (40 °C, 350 atm pressure), and suggested that propolis contains vitamin C (Kobayashi *et al.*, 2001). Collectively, these results suggest that propolis has both an antioxidant effect and a free-radical scavenging effect.

Figure 3 shows the tendency for inhibition growth of the Sarcoma 180 tumor by combined administration of water-soluble propolis and irradiation. Similar antitumor effects have been reported previously; hence, Rodrigo *et al.* showed an effect of water-soluble propolis on tumorigenesis of colorectal cancer, and showed that water-soluble propolis regulates DNA damage, using a Comet assay (Rodrigo *et al.*, 2005). It has also been reported that propolis and its components, including flavonoids, aromatic carboxylic acids and esters, prevent oncogenesis (Rao *et al.*, 1993; Bazo *et al.*, 2002). Matsuno *et al.* showed that PRF-1 extracted from propolis has toxicity for human hepatocellular carcinoma (Matsuno *et al.*, 1997), and Kimoto *et al.* showed that propolis suppresses FeNTA-induced renal adenocarcinoma in CD-1 and ddY mice (Kimoto *et al.*, 2000). Clinical treatment methods using propolis have yet to be clearly established, but many pathological studies have shown biological effects of propolis (Grunberger *et al.*, 1988; Mitamura *et al.*, 1995; Mitamura *et al.*, 1996; Velikova *et al.*, 2000; Banskota, 2001; Kimoto *et al.*, 2001; Akao *et al.*, 2003). It has been reported that leukocytes and $CD8^+$ and $CD4^+$ cells are significantly increased in water-soluble propolis-treated mice (Ivanovska *et al.*, 1995), and propolis and its components have been shown to have mobility and bactericidal properties *in vivo* and *in vitro*, while enhancing tumorigenic activity and producing factors activating IL-1, TNF and lymphocytes in mammals (Dimov *et al.*, 1991; Dimov *et al.*,1992; Ivanovska *et al.*, 1995; Murad *et al.*, 2002). It is well known that irradiation of tumors suppresses tumor growth: irradiation has a direct effect on tumor DNA, thereby suppressing cell growth and inducing apoptosis (Neal *et al.*, 1981), and can delay tumor growth by an oxygenic effect (reoxygenation) on tumor blood vessels (Van Putten *et al.*, 1958). The combined antitumor effects of propolis and irradiation-inhibited tumor growth appear to be stronger than the effects of each treatment alone.

In conclusion, prior administration of water-

soluble propolis enhances the immune systems and suppresses irradiation-induced damage to hemocytes in the peripheral blood. Water-soluble propolis also shows an antioxidant effect in reducing irradiation-induced free-radical damage, and can inhibit the growth of sarcoma cells, with increased inhibition occurring in combination with irradiation.

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