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Immunomodulation of NK Cell Activity by Red Ginseng Acidic Polysaccharide (RGAP) in Ovariectomized Rats

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Abstract : The *in-vitro* immunomodulatory function of murine natural-killer (NK) cells induced by red-ginseng acidic polysaccharide (RGAP) in ovariectomized (OVX) rats was examined in this study. The IL-2-induced NK cell activity was significantly decreased in the OVX rats compared to the sham groups, but the normally induced NK cell activity was not. RGAP, however, increased the NK cell activity in both groups, and this effect involved iNOS expression. The inhibition of iNOS activity did not increase the NK cell cytotoxicity by RGAP in the OVX rats. The data that were obtained also demonstrated that the expression of iNOS was increased in the spleen of the OVX rats. These results indicate that RGAP increases the tumoricidal activity of the NK cell in the OVX rats, which is a primed or activated state of innate immune cells resulting from the changes in cytokine production induced by estrogen-deficient stress. Therefore, RGAP has a synergistic effect on the NK cell activities, which are regulated by the iNOS signals in OVX rats. This suggests that RGAP is useful for potential therapeutic strategies as a nutrient in regulating the NK cells in OVX rats.

Key words : red-ginseng acidic polysaccharide, ovariectomized, NK cell, iNOS, rat

INTRODUCTION

Aging plays an important role in many biological functions within a body, and it is the main predictor for osteoporosis. With advancing aging and particularly with post-menopausal conditions, the activity of osteoclasts, cells that break down bone through bone resorption, is greater than that of osteoblasts, cells responsible for bone formation, in women. This ultimately gives rise to osteoporosis.[1)] Aging is also associated with a decline in immune function, known as immunosenescence, that contributes to the increased susceptibility to infection, cancer, and autoimmune diseases observed in humans.[2,3)] Aging-related alterations of the cellular components of innate immunity might therefore be involved in the impairment of adaptive immunity observed in the elderly.[2)]

Natural killer (NK) cells are an important component of the innate immune system and mediate cytolytic activities against tumors and virus-infected targets. Recently increasing evidences indicate that NK cells may play a pivotal role in some female predominant diseases or in normal physiological conditions. Some studies suggest that estrogen may be involved in the regulation of immunocompetence in immune system and it modulates activities of NK cells.[4)] Baral *et al.* also found that estrogen reduced NK cell cytotoxicity against tumor targets but this effect was dependent on duration of exposure to estrogen.[5)]

The root of *Panax ginseng* C.A. Meyer (Araliaceae) is one of the most popular natural tonics used in Asian countries. Water extracts of ginseng have shown antitumor activity against some kinds of tumor cells in mice and have inhibited the incidence of lung cancers induced by a wide range of carcinogens.[6,7)] In epidemiologic studies, ginseng intake reduced the incidence of human cancers.[8)] In a previous study, we isolated red ginseng acidic polysaccharide (RGAP) from Korean red ginseng and found it to induce a proliferation of spleen cells, to decrease the antibody-forming cell response to sheep red blood cells, and to stimulate nitric oxide (NO) production in murine peritoneal macrophages in vivo.[9)] In addition, RGAP has recently been found to show immunomodulating and anticancer properties in a murine-transplanted tumor cell model.[10)] Although the favorable effects of RGAP have been reported and evaluated, the effects of RGAP on activity of NK cells in osteoporosis have not

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been evaluated. The estrogen-deficient ovariectomized (OVX) osteoporosis model is useful for evaluation of osteoporotic drugs because several bone-related parameters are clearly decreased by ovariectomy within 10 to 14 weeks after the operation. In this model, we examined the effects of RGAP on the NK cell function in ovariectomized rats and identify the possible involvement of iNOS that may mediate the regulatory effect of NK cell.

MATERIALS AND METHODS

1. Preparation of red ginseng acidic polysaccharide

Red ginseng made by steaming and drying fresh root of *Panax ginseng* C.A. Mayer was cut to mill. Powdered red ginseng was percolated with 5 volumes of 85% ethanol to extract off ethanol-soluble materials. The remaining residues were repercolated with 5 volumes of distilled water, and the water-soluble extracts were concentrated with a vacuum evaporator. The concentrate was dialyzed against running tap water for 7 d to completely cut off small molecules of less than 15 kDa. Four volumes of absolute ethanol were added to precipitate the polysaccharide in the inner dialysate. The precipitate was dried in a vacuum drying oven, and was finally used as a red ginseng acidic polysaccharide (RGAP). The chemical composition of RGAP was 56.9% acidic sugars and 28.3% neutral sugars as determined by carbazole assay and phenol-sulfuric assay respectively.[11] The protein content of RGAP was below 0.1% as determined by the Lowry method. Less than 0.006 EU (endotoxin units) of endotoxin was present in 1mg of RGAP as tested by Limulus amebocyte lysate assay. This level of endotoxin did not affect the experimental results obtained by RGAP.

2. Experimental animals and diets

Female Sprague–Dawley (SD) rats (12 weeks old, SPF) were purchased from Samtaco Bio Korea (Osan city, Kyunggido, Korea). The rats were housed at 23 ± 3°C with 55% ± 15% humidity. The temperature and light/dark cycle (12-h light, 12-h dark) were automatically controlled in the animal care facility of the Sungkyunkwan University, Suwon, Korea. Commercial laboratory rat food (Harlan Co. Ltd.) was given to the rats ad libitum. After a one-week acclimatization period, the SD rats were divided into five per groups, including one group of sham-operated rats and other groups of ovariectomized (OVX) rats. The surgery for ovariectomy was performed under anesthesia using a muscular injection of 40 mg/kg ketamine. We confirmed that the ovariectomy significantly decreased (t-test, $p<0.05$) bone mineral density at 10 weeks after surgery, when experimental treatments were started. Animal care and all experimental protocols were performed following the Institute for Laboratory Animal Research (ILAR) guideline.

3. Chemicals

Unless stated otherwise, all chemicals were purchased from the Sigma Chemical Co. (St Louis, MO). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). The XTT {2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt} cell viability assay kit was purchased from WelGENE (Daegu, South Korea). All the tissue culture reagents and RGAP were assayed for any endotoxin contamination using the Limulus lysate test (E-Toxate, Sigma), and the endotoxin levels were found to be < 10 pg/ml.

4. NK cell-mediated tumoricidal activity

A modification of the method reported by Mosmann *et al.* was used.[12] The spleens were aseptically removed and dissociated into a single-cell suspension in a culture medium. The concentration was adjusted to 2×10^5 cells/mL. The culture medium was RPMI 1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL) (RPMI-FBS). Spleen cells from the shame and OVX rats were tested as effector cells and YAC-1 mouse lymphoma cells (ATCC, Rockville, MD) were used as the target cells. The NK cell assays were carried out in 96-well v-bottom plates at effector/ target cell ratios 50:1 with 1×10^4 of the target cells in a final well volume of 200 μL at 37°C for 6 h in a 5% CO_2 humidified incubator. After incubation of cells for 24 h, 20 μL of phenazine methosulphate (PMS; electron-coupling reagent) and 25 μL of XTT was added to each well. The cells were further incubated for 3 h to allow XTT formazan production. The absorbances were determined with a microplate reader at a test wavelength of 450 nm and a reference wavelength of 690 nm.

5. Western blot analysis

The amount of iNOS was measured by Western blot analysis. Rats were immediately sacrificed and isolate spleen. After homogenized of spleen tissue and lysed in sodium dodecylsulfate polyacrylamide gel electrophoresis buffer. Protein concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of each sample was electro-

phoresed on 10% sodium dodecylsulfate polyacrylamide gel electrophoresis gels and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline/non-fat Tween for 1 h. The membranes were incubated with primary antibody against iNOS for 24 h. They were then washed with Tris-buffered saline/non-fat Tween once for 15 min and three times for 5 min and incubated with secondary ALP-conjugated anti-rabbit antibody for 1 h. The membranes were washed again as described above. Autoradiography was carried out using an enhanced chemiluminescence kit (Amersham Bioscience).

6. Statistical analysis

Each experiment was repeated at least two times, and the results of one representative experiment are shown. The results were expressed as means±S.E.M. and analyzed via ANOVA. The significant values are represented by an asterisk (*$p<0.05$ and **$p<0.01$).

RESULTS AND DISCUSSION

The most characteristic function of NK cells is non-MHC restricted killing of target cells, such as tumor, and thus NK cells are important for maintaining health in body.[13] It has been reported that aging attributed to various factors of NK cells. For example, NK cells exhibited decreased responsiveness to its positive modulator, IL-2, in aged group,[14] and this explains the high frequency of cancer and infectious diseases with aging. Osteoporosis is one of the aging-related diseases.[15] With advancing aging and particularly after menopause in women, the activity of osteoclasts, cells that break down bone, is greater than that of osteoblasts, cells that build bone. In this study, we determined the cytotoxicity and responsiveness to IL-2 of NK cells in OVX. As shown Fig. 1 there was no difference in NK cell cytotoxicity between sham control and OVX groups, while its responseveness to IL-2, such as LAK activity, was decreased in OVX groups. These results suggest that osteoporosis patient is susceptible in pathogen infection, because osteoporosis may cause changes in the innate response to IL-2 and cytotoxicity of NK cells. Recently it has been reported that NK cell cytotoxicity could be regulated by sex hormones. Hao *et al.* found that there was an inhibition of specific lysis of Yac-1 target after a long-term exposure of the NK cells to 17β-estradiol.[16] In addition, several data in animal model studies also provided direct evidence that chronic administration of high doses of 17β-estradiol to mice resulted in a decreased activity of NK cells.[17] Thus, estrogen imbalance level caused change of NK cell cytotoxicity and the low NK activity induced by IL-2 was found to be a predictor of morbidity for various infection and cancer in osteoporosis.[18]

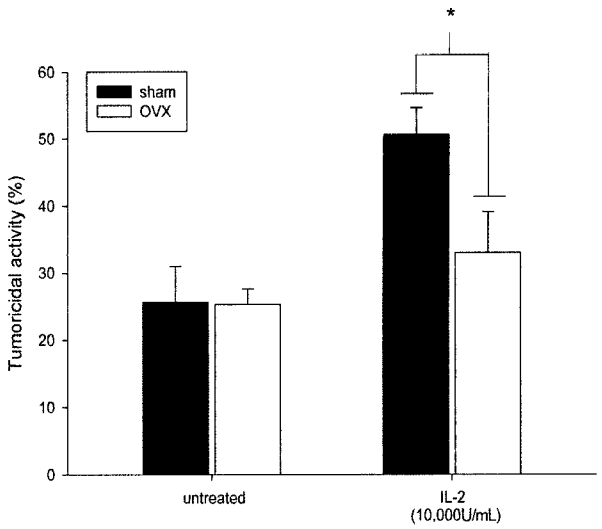

Fig. 1. NK cell mediated-tumoricidal activity and responsiveness to the positive modulation with IL-2. Splenocytes were cultured in the absence or presence of IL-2 (10,000u/ml) for 4 h in sham and OVX gropus, respectively. The turmoridical activity was examined at E:T ratio of 50:1. The data represents the means±S.E.M. of quadruplicate experiments. *: Significantly different from control (no treatment); *$p<0.05$.

The in vitro treatment with RGAP resulted in a significant enhancement in NK cell activity in OVX rats (Fig. 2B). In the previous study, we found that the treatment of non-saponin red ginseng fraction significantly increased NK cell cytotoxicity at 100~300 μg/mL,[19] and the present data demonstrate that RGAP also has a significant effect on NK cell activity at 1 μg/mL in normal groups and at 1~100 μg/mL in OVX (Fig. 2A, B). As shown in Fig. 2A and B, RGAP enhanced tumoricidal activity of NK cells only at 1 μg/mL, which is similar to the previous reports documenting the efficacy of about non-saponin red ginseng fraction in murine peritoneal macrophages.[19,20] At present time, we did not know why tumoricidal activity is not induced by RGAP at high concentrations. It is plausible that cells could be desensitized by RGAP at high concentrations and subsequently cause the modification of receptor molecules.

The cytokines produced by NK cells such as NO and IFN-γ affect their survival or partially mediate their cytotoxic function to modulate host immune responses.[21,22] In

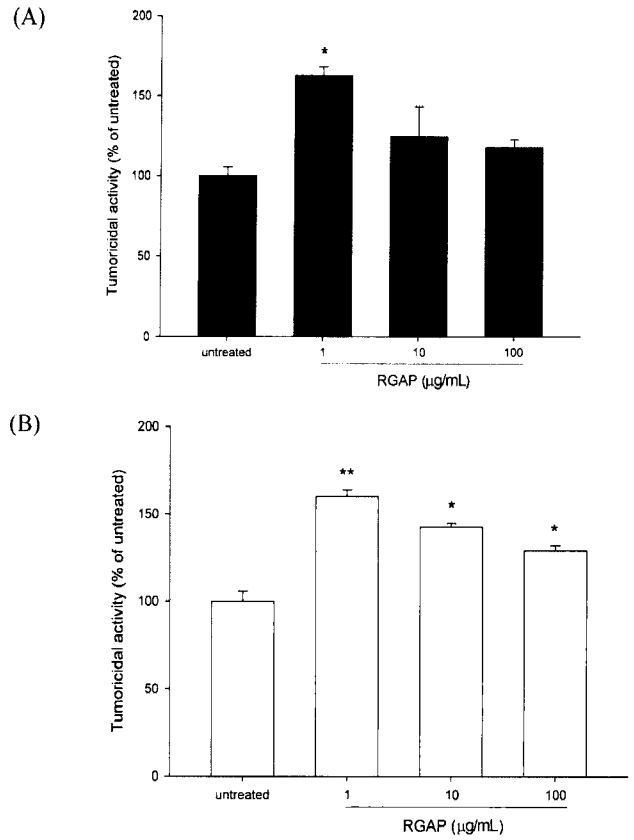

Fig. 2. In vitro effect of RGAP on the NK cell-meidated tumoricidal activity. Splenocytes were cultured with various RGAP doses (1~l00 μg/mL) for 4 h in sham (A) and OVX group (B), respectively. The turmoridical activity was examined at E:T ratio of 50:1. The data represents the means±S.E.M. of quadruplicate experiments. *: Significantly different from control (no treatment); *p<0.05 and **p<0.01.

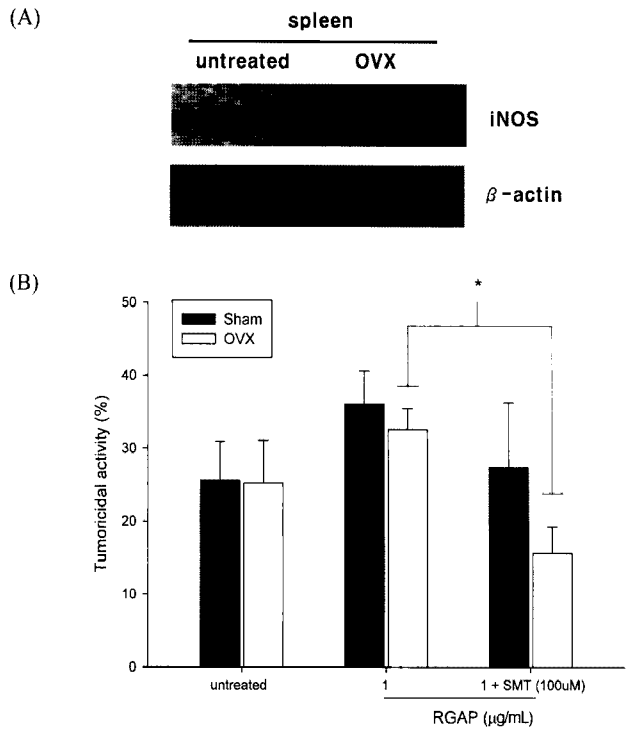

Fig. 3. Effect of iNOS expression in spleen from OVX rats (A) and iNOS inhibitors on RGAP-induced NK cell-meidated tumoricidal activity (B). Spleen tissue lysate were determined by Western Blot assay and splenocyte (5×10^4 cells/well) were treated with SMT (100 μM) in the absence or presence of RGAP for 4 h. The NK cell-meidated tumoricidal activity was examined at E:T ratio of 50:1. The data represents the means±S.E.M. of quadruplicate experiments. *: Significantly different from the SMT-untreated groups; *p<0.05.

this study, we showed that there is a significant increase in iNOS expression in splenocyte from OVX rats (Fig. 3A). To examine whether the effect of RGAP on NK cell cytotoxicity is mediated by NO and which NOS is involved in this effect, the cells were treated with RGAP (100 μg/mL) in the absence or presence of S-methylisothiourea (SMT), preferential inhibitor of iNOS. As shown in Ffg. 3B, it appears that iNOS expression was involved in NK cell activity in OVX. In addition, inhibition of iNOS slightly suppressed the NK cell cytotoxicity in sham groups but not significant compared to that of OVX groups. These data imply that the estrogen-deficient stress activates innate immune cells such as NK cells. Thus it is speculated that RGAP and cytokines produced by these cells could exert cooperative effects on NK cell activities in OVX state. In previous study, RGAP alone had no effect on the function of macrophages for killing tumor cells. However, a combination of RGAP and recombinant IFN-γ (rIFN-γ) enhanced their killing of tumor cells.[9] RGAP treatment also did not change the production of IL-1, NO, TNF-α, whereas the sequential treatment with rIFN-γ and RGAP yielded significantly increased production of TNF-α, NO and IL-1 in peritoneal macrophages. It was reported that IFN-γ was a major product of murine NK cells during pregnancy and its production was enhanced by a long-term exposure of cells to estradiol.[17,23] Therefore, we could postulate that hormonal imbalance such as estrogen-deficiency changed the secretion of various cytokines of NK cells. Moreover, the RGAP exerted cooperative influence on such cytokines for NK cell activities, and it was regulated by iNOS.

Our study suggested that RGAP have a possibility as a potential immunotherapeutic agent for osteoporotic patients. Though we do not know whether iNOS expression is involved in NK cell activities, our data demonstrate that

the effect of RGAP on NK cell-mediated tumoricidal activity is regulated by iNOS expression and RGAP provides a second signal for synergistic induction of NK cell activities in OVX rats. The precise mechanism of estrogen in modulating NK cells and the exact role of NK cells in the diseases remain unclear. In addition, the exact explanation for modulation of NK activity by RGAP in OVX rats is not known yet. Therefore we need to examine further the effects of RGAP on the expression of iNOS and the level of cytokines, which are may necessary for activation and maturation of NK cells.

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