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Immunomodulatory Effects of Non-saponin Red Ginseng Components on Innate Immune Cells

Eun-Hwa Sohn*, Jihye Kim*, Hyesook Choi***, Hye-Jin Park***, Byung-Oh Kim**, Dong-Kwon Rhee*** and Suhkneung Pyo***, #**

**Department of Herbal Medicine Resource, Kangwon National University, Samcheok, Gangwondo, 245-711*
***Department of Applied Biology, Kyungpook National University, SangJu, Gyeongsangbuk-do, 742-711*
****College of Pharmacy, Sungkyunkwan University, Suwon, Gyunggi-do, 440-746, Republic of Korea*
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Abstract : Macrophages and NK cells play an important role in the first line of immunologic effects against tumor cells. We therefore assessed the effects of non-saponin red ginseng components (NSRG) on NK cell and macrophage tumoricidal activities, and the mitogen-stimulated lymphoproliferative response. When NK cells were treated with various concentrations of NSRG (100-300 ug/ml) for 4 h, tumoricidal activity was significantly increased. However, NSRG had effects on tumoricidal activity of macrophages at low concentration (1 ug/ml), which was not related to the production of nitric oxide. The mitogen response of lymphocytes to LPS and ConA in the spleen did not show significant differences between the control and NSRG-treated cells, whereas LPS-induced blastogenesis was slightly increased at 100 μg/ml ($p < 0.05$). These results suggest that NSRG has differential effects on innate immune response and could be useful as immunotherapy for cancer treatment.

Key words : non-saponin red ginseng, natural killer cells, tumoricidal activity, immunotherapy

INTRODUCTION

White ginseng (Ginseng Radix Alba) is produced by air-drying the root, while red ginseng (Ginseng Radix Rubra) is produced by steaming the root followed by drying. During the heat processing for preparing red ginseng, it has been found to exhibit changes in ginsenosides composition. Ginsenosides Rh_2, Rh_4, Rs_3, Rs_4 and Rg_5 found only in red ginseng, have been known to be hydrolyzed products derived from original saponin by heat processing and responsible for inhibitory effects on the growth of cancer cells through the induction of apoptosis[1]. Additionally, one of non-saponin components, panaxytriol, was found to be structually transformed from polyacetylenic alcohol (panaxydol) showing cytotoxicity during the preparation of red ginseng and also maltol, antioxidant maillard product, from maltose and arginyl-fructosyl-glucose, amino acid derivative, from arginine and maltose[2].

Hiromichi *et al*. suggest that pharmacological actions of ginseng roots may be derived from its non-saponin fractions as well as ginsenosides[3]. Several publications have reported that acidic polysaccharides from non-saponin red ginseng components (NSRGs) are known to prevent obesity or hyperlipemia by enhancing immunity against cancer, inhibiting lipolysis and lowering intestinal absorption of cholesterol or fat[4,5]. Larina L *et al*. also showed that saponin-free fraction from Ginseng significantly improves an oxygen-dependent anti-microbial function of neutrophils[6].

The innate immune system represents the first line of defense against microorganisms as it promptly initiates a local inflammatory reaction. Macrophages and natural killer (NK) cells are an important components of the innate immune system and mediate the cytolytic activities against tumor and virus-infected targets[7,8]. However, immunomodulatory effects of non-saponin gingseng fraction components on innate immune system are still unknown. Therefore, this study examined the effect of non-saponin Ginseng components on the NK cell, macrophage and lymphocyte blastogenesis activities.

To whom correspondence should be addressed.
(Tel) +82-31-290-7713; (Fax) +82-31-292-8800
(E-mail) snpyo@skku.ac.kr

MATERIALS AND METHODS

NSRG preparations

Six-year Korean red ginseng was obtained from the Korean Society of Ginseng. 100 g of 6-year red ginseng was extracted three times with 1000 ml of MeOH for 4 h under reflux. The resulting extract 13.5g, was subjected to filtration and concentration. 10 g of this was dissolved in 100 ml of water and was extracted 3 times with 100 ml ether. The supernatant was dehydrated by Na_2SO_4, and was decompressed for concentration within 40°C yielding 830 mg of the powder (Fig. 1). The water saturated fraction was extracted three times with 100 ml ethylacetate and was separated with ethylacetate (EtOAc fr.) and water. The water fraction was extracted three more times with 100 ml n-BuOH, filtered and concentrated yielding a 1.4 g saponin fraction. Finally, 10.2 g of the H_2O fraction was obtained by the decompression followed by the condensation of the remaining supernatants (Fig. 1).

Animals and Chemicals

Male C57BL/6 mice (7 weeks old) were obtained from the Charles River Breeding Laboratories (Japan). The animals were randomly distributed into five per group. During the experimental period, the animals were maintained at 23±1°C, 55±5% humidity, 10-18 circulation/hour and 12 hrs cycle of light/dark. The animals were given access to food and water ad libitum. 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 NSRG 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.

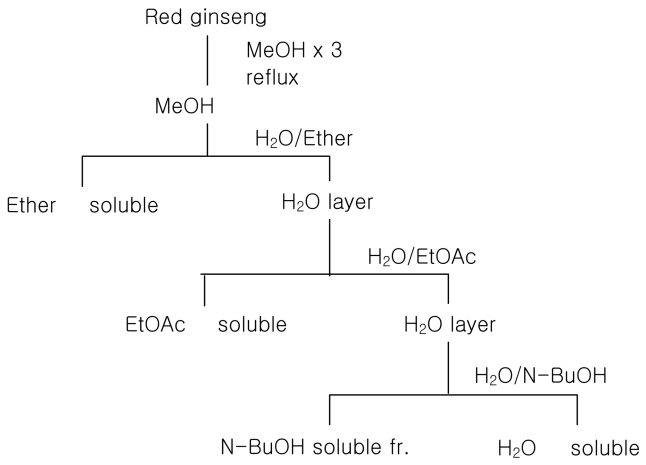

Fig. 1. Various solvent fraction of red ginseng.

Macrophage-mediated tumoricidal activity

The assay for macrophage tumoricidal activity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay adapted from Mosmann[9]. Briefly, macrophages (1×10^5cells/well) incubated in either medium alone or in medium supplemented with various doses of NSRG for 24 h in 96-well plates. Macrophages were washed with RPMI-FBS (RPMI 1640 containing 10% heat-inactivated FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml)) to remove NSRG and then co-incubated with B16 melanoma cells (1×10^4cells/well; effector:target cell ratio of 10:1) for 24 h. The enzyme activity of viable cells was measured by addition of MTT to each well. After additional incubation for 4 h, the amount of formazan was determined by absorbance at 540 nm using a microplate reader. (Menlo Park, CA, USA). Cytotoxic activity is expressed as the percentage of tumor cytotoxicity by the following formula : [1-{OD of (target cells + macrophages) - OD of macrophages}/OD of target cells]×100. OD of target cells is the optical density of B16 melanoma cells and OD of macrophages is the optical density of macrophages.

Nitrite determination

The cells were treated with various doses of NSRG for 24 h and the accumulation of nitrite in culture supernatants was measured using the assay system described by Ding *et al.* [10]. 100 μL aliquots of culture supernatants were mixed with an equal volume of Griess reagent (mixture at 1:1 of naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4) and incubated at room temperature for 10 min. Nitrite concentration was cal- culated from a $NaNO_2$ standard curve.

Mitogen-induced cell proliferation (XTT assay)

A modification of the method reported by Mosmann *et al.* was used[9]. The spleens were aseptically removed and dissociated into a single-cell suspension in a culture medium. The concentration was adjusted to 2×10^5cells/ml. The culture medium was RPMI-FBS. Cells (5×10^5cells/well) were incubated with various NSRG concentrations (100-300 μg/ml) in the presence or absence of the mitogens as follows: concanavalin A (ConA) at 4 μg/ml for T cell activation and lipopolysaccharide (LPS) at 10 μg/ml for B

cell activation. After incubation of cells for 24 h, 20 μl of phenazine methosulphate (PMS; electron-coupling reagent) and 25 μl of XTT [2,3-Bis(2-methoxy-4-nitro-5- sulfophenyl)-2H-tetrazolium-5-carboxanilide] 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. Tumoricidal activity of NK cell was measured by XTT assay in place of MTT assay and expressed as the same calculation method as described in material and methods about macrophage tumoricidal activity.

Assessment of NK cell cytotoxicity

The NK cell cytotoxicity was analyzed on a flow cytometer (Bio-Rad Laboratories, Hercules, CA)[11,12]. Spleen cells from the male C57BL/6 mice were tested as effector cells and EGFP-transfected YAC-1 stable cells were used as the target cells. The NK cell cytotoxicity assays were carried out in 6-well plates at effector/target cell ratio 50:1 with 1×10^4 of the target cells in a final well volume of 2 ml for 4 h at 37°C in a 5% CO_2 humidified incubator. Propidium iodide (PI)(Sigma, MO, USA) at a concentration of 100 μg/ml was added to each tube including effector and target cells, and then incubated at room temperature for 15 min. PI was used to stain dead cells. Linear amplification of the forward and side scatter (FSC/SSC) signals, as well as logarithmic amplification of EGFP (Green fluorescent protein; Green signal) and PI (Propidium iodide; Red signal) emission in green and red fluoresence were obtained. Calculation of percent lysis of target cells by the proposed formula: % lysis={UR/(UR + LR)}×100 without using the autodamaged (GFP-/PI+)/ potentially lysed target cells(GFP+/PI-) where UR is upper right quadrant region and LR is lower right quadrant region in cytogram.

Statistical analysis

The data are represented as a mean ± SE. The statistical difference between the groups was determined using a one-way analysis of variance (ANOVA) with a Student-Newman-Keuls test. A P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Since activation of macrophages plays an important role in the host defense mechanism and NO is related to cytotoxic function of macrophages against a variety of tumors and microorganisms[13-15], we examined the effects of NSRG on macrophage-mediated tumoricidal activity and NO production. NO was produced in high amounts by iNOS in activated macrophages, and then excessive formation of NO mediates the bactericidal and tumoricidal actions of macrophages.

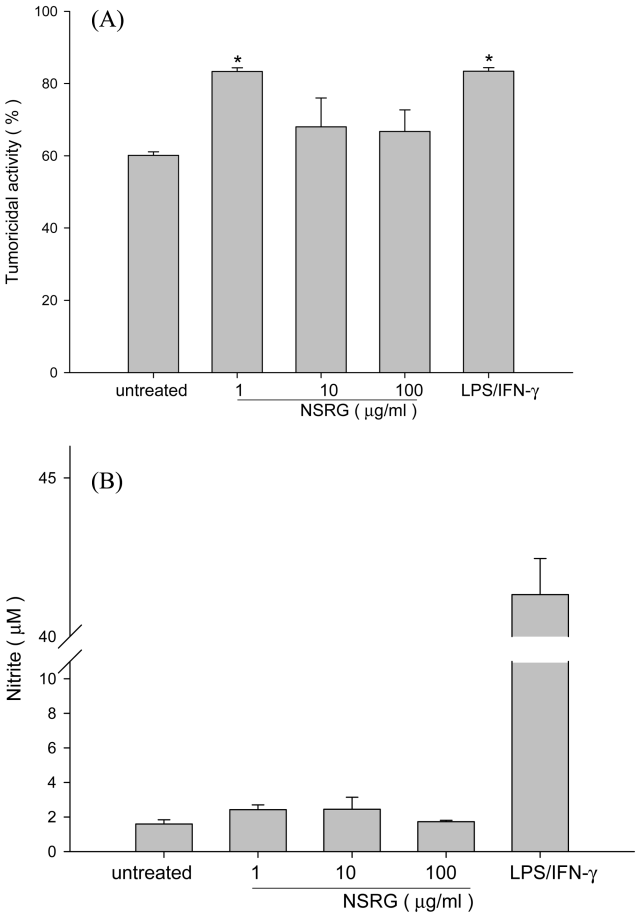

Fig. 2. Effects of NSRG on tumoricidal activity (A) and nitric oxide production (B) in RAW264.7 cells. The cells were co-cultured with target cells (B16) at an effector/ target ratio of 10:1 for 24 h in the presence or absence of various doses of NSRG concentration (1-l00 μg/ml). Macrophage tumoricidal activity was measured as described in materials and methods. The formazan formation of macrophages was determined by MTT assay. Cell density was measured at 540 nm. Culture supernatants were collected and the levels of nitrite were measured as described in materials and method. The data represent the mean ± SE of quadruplicate experiments. *: Significantly different from control (no treatment); *p<0.05.

To examine whether NSRG treatment stimulates the tumoricidal activities of macrophages against target tumor

cells, we co-cultured Raw264.7 cells with B16 cells for 24 h. B16 tumor cells were used as target since they are either TNF-α or NO sensitive. As shown in Fig. 2, NSRG enhanced tumoricidal activity of macrophage only at 1 μg/mL, which is similar to LPS/IFN-γ treatment. At present time, we did not know why tumoricidal activity is not induced by NSRG at high concentrations. It is plausible that cells could be desensitized by NSRG at high concentrations and subsequently cause the modification of receptor molecules. In fact, LPS has been known to induce the desensitization in various experimental systems[16]. The present data show that the production of NO was not induced in NSRG-treated macrophages, suggesting that NO are not involved in the tumoricidal activity of macrophage induced by NSRG. Although other cytotoxic molecules such as TNF-α, IFN and IL-1 were not examined in this study, it is probable that the tumoricidal activity of macrophages could be mediated by other cytotoxic molecules but not nitrix oxide.

In the next experiment, to examine the effect of NSRG on the mitogen-stimulated lymphoblastogenic responses, XTT assays were performed by stimulating spleen cells with the mitogens, ConA or LPS, after exposure to NSRG for 24 h. As shown in Fig. 3, splenic lymphocyte blastogenesis was not affected by NSRG alone. In addition, blastogenesis was not altered in mitogen-treated cells as compared to control, whereas LPS-induced blastogenesis was slightly increased at 100 μg/ml ($p < 0.05$).

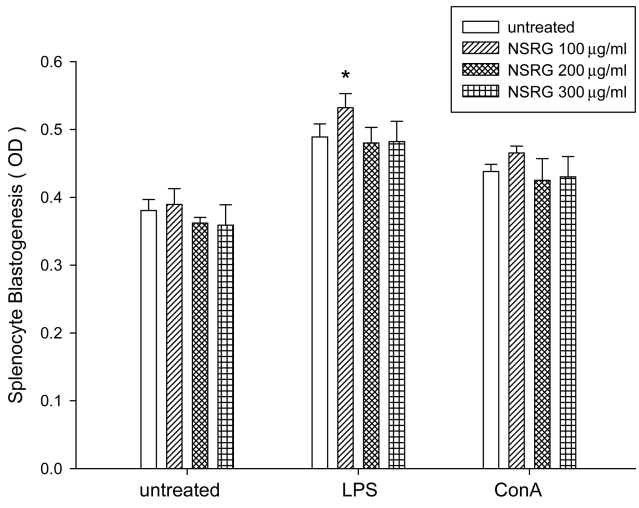

Fig. 3. Effect of NSRG on the lymphocyte blastogenic function. The splenocytes were incubated with various NSRG concentrations (100-300 μg/ml) in the presence or absence of Con A and LPS for 24 h, and the absorbance was measured at 570 nm. The data are expressed as a mean ± SE of quadruplicates of a representative experiment. *: Significantly different from control (no treatment); *$p<0.05$.

Culturing the splenocytes with NSRG for 4 h, the lysis of Yac-1 cells were analysed by flow cytometry and resulted in the enhancement of NK cell activity at 100 and 300 μg/ml. Although there is a big difference between % lysis by treated cells and % lysis by untreated cells, NSRG increased the NK cell activity by 182% at 100 μg/ml and the activity was further increased (273%) with increasing the NSRG concentration to 300 μg/ml (Fig. 4(A)). To further confirm these effects, XTT assay was used to measure the NK cell activity. As shown in Fig. 4(B), the similar results were observed. There were no significant changes in the number of NSRG-treated lymphocytes with the as compared to that of nontreated cells (data not shown). These findings provide further evidence for the concept that NSRG can activate the cytolytic activity of NK cells without affecting the cell viability. Overall, these findings clearly demonstrate that NSRG could induce the activity of NK cells.

In summary, the present data suggest that NSRG induces the activation of NK cells, whereas it had no effect on the T-lymphocyte proliferation, at same concentration. In addition, NSRG treatment resulted in the increase of tumoricidal activity of macrophages at low concentration (1 μg/ml), which is not related to NO production.

Several studies have shown that a single administration of a biological response modifier (BRM) significantly enhanced the NK cell activity[17,18]. Accordingly, the augmentation of NK activity by different BRMs has been the focus of many investigations. Based on our findings, NSRG has a potential immunomodulating effects on innate immune system. In addition, the dose used and the data presented might be expected to expand the database on the effect of NSRG on the innate immune system.

Until now, many researchers recognized that one of the most important medicinal components of red ginseng is saponin, and the content of saponin which are believed to be main principles of pharmacological actions of red ginsengs[19-21]. However, in the present study, we suggest that non-saponin red ginseng component has immunomodulating effects. The exact explanation of the way how NSRG modulate immune responses is not known. Additional work may be necessary to investigate the interaction of immune system activation and to clarify how this activation occurs and to what extent it occurs in vivo.

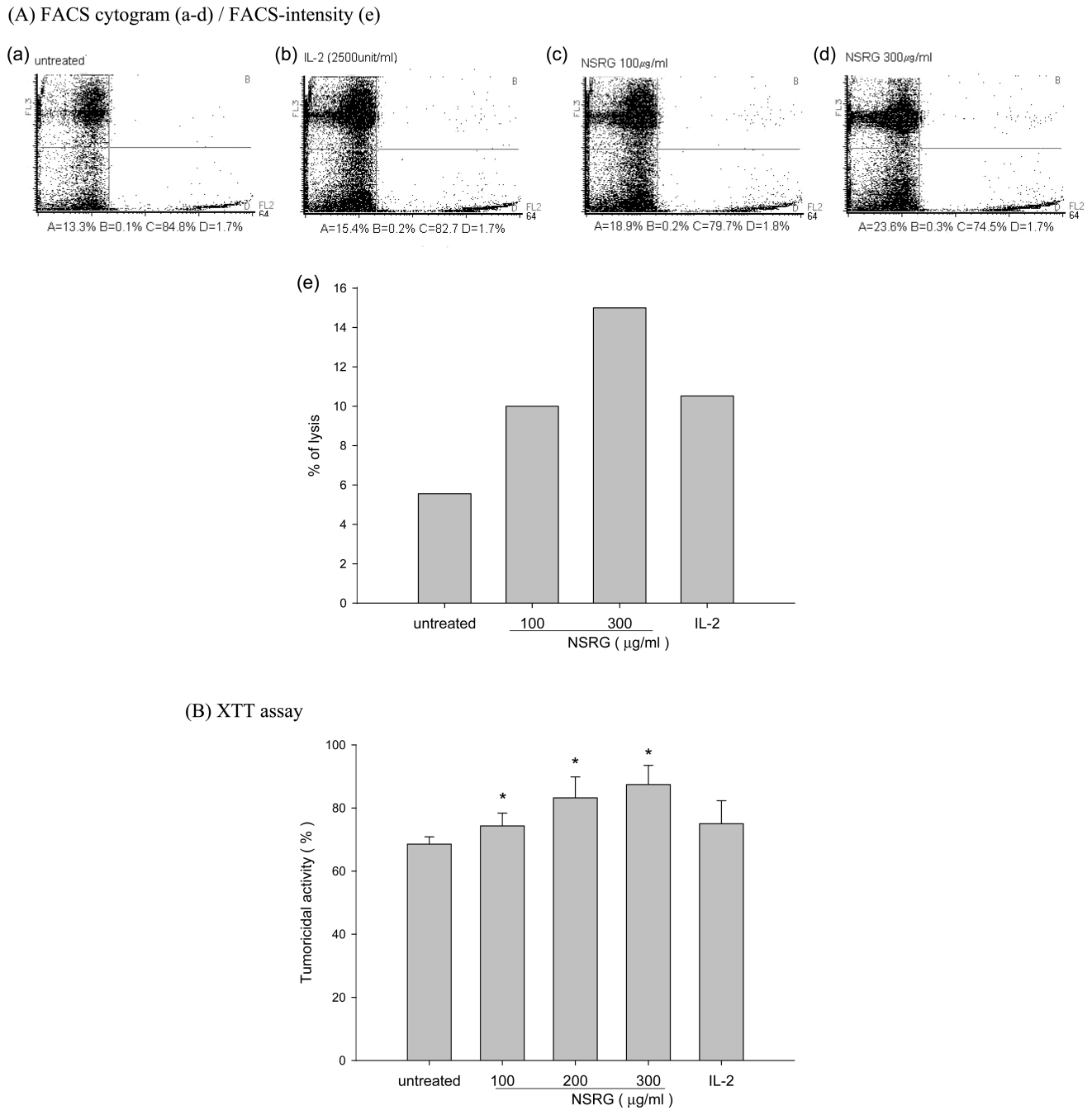

Fig. 4. Effect of NSRG on the NK cell cytotoxicity. Splenocytes were cultured with various doses of NSRG (100-300 μg/ml) for 4 h. The NK cell cytotoxicity was examined at effector:target ratio of 50 : l by FACS (A) and XTT assay (B), respectively. The FACS data were also presented as a bar graph (a-e). The data represent the mean ± SE of quadruplicate experiments. *: Significantly different from control (no treatment); *p<0.05.

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