J. Ginseng Res.
Vol. 33, No. 4, 278-282 (2009)

Enhancement of Murine NK cell Activity *in vitro* by Red Ginseng Acidic Polysaccharide

Hye-Sook Choi[1], Eunwha Sohn[2], Dong-Kwon Rhee[1] and Suhkneung Pyo[1]*

[1]*School of Pharmacy, Sungkyunkwan University, Suwon, Kyunggi-do 440-746, Korea*
[2]*Department of Herbal Medicine Resources, Institute of Bioscience and Biotechnology, Kangwon National University, Samcheok, Gangwon-do 245-711, Korea*
(Received November 19, 2009; Revised December 13, 2009; Accepted December 14, 2009)

Abstract : The *in-vitro* immunomodulatory function of the activity of murine natural-killer (NK) cells induced by red-ginseng acidic polysaccharide (RGAP) was examined. RGAP induced the significant enhancement of NK cell activity against the Yac-1 tumor cells. The treatment of splenocytes cultured with RGAP for 16 h resulted in a significant increase in NK activity at the E:T ratio of 100:1, and in a 239 and 250% increase at 10 and l00 μg/ml, respectively. We also demonstrate that RGAP treatment increased the production of interferon (IFN)-γ (17-125%) and tumor necrosis factor (TNF)-α (15-100%), suggesting that the increase in NK cell cytotoxicity could be due to the enhancement of the NK cell production of both cytokines. In addition, RGAP had a stimulating effect on lymphocyte proliferation in the presence of mitogens. Overall, these results suggest that RGAP has an immunopotentiating effect on NK cells, which can support the development of clinical studies on RGAP.

Key words : red ginseng, NK cell, cytotoxicity, cytokines

INTRODUCTION

Natural killer (NK) cells are an important component of the innate immune system and mediate the cytolytic activities against tumor and virus-infected targets. In addition to the cytotoxic activity, there are increasing indications that NK cells can secrete a variety of cytokines and cytotoxic factors.[1-3] Several studies have shown that the NK system is usually impaired in cancer patients.[4-6] As a result, recent attention has focused on determining new immunotherapeutic approaches for treating cancer by increasing the host anti-tumor response through the augmentation of the NK cell activity.

The root of *Panax ginseng* C.A. Meyer (Araliaceae) is one of the most popular natural tonics which used in Asian countries. Water extracts of ginseng have shown antitumor activity in some kinds of tumor cells in mice[7] and have inhibited the incidence of lung tumors induced by a wide range of carcinogens.[8] In epidemiologic studies, ginseng intake reduced the incidence of human cancer.[9] However, the active substance and its mechanism of action remain unclear. In a previous study, red ginseng acidic polysaccharide (RGAP) from Korean red ginseng was found to decrease the antibody-forming cell response to sheep red blood cells and to stimulate nitric oxide production in murine peritoneal macrophages *in vivo*.[10,11] RGAP has been also found to show immunomodulating and anticancer properties in a murine-transplanted tumor cell model.[12,13] Moreover, we demonstrated that RGAP treatment increased the production of interleukin-1 (IL-1), IL-6, and nitric oxide by macrophages.[13] In the present study, we examined the effect of RGAP on the NK cell activity.

MATERIALS AND METHODS

Animals and reagents

Male C57BL/6 mice (7 weeks old) were obtained from the Charles River Breeding Laboratories (Atsugi, Japan). 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). 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.

* Corresponding author. E-mail: snpyo@skku.edu
Phone: +82-31-290-7713, Fax: +82-31-290-7733

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 days 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.[14] 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 1 mg of RGAP as tested by Limulus amebocyte lysate assay. This level of endotoxin did not affect the experimental results obtained by RGAP.

RGAP-induced cell proliferation

A modification of the method reported by Mosmann et al. was used.[15] The spleens were aseptically removed and dissociated into a single-cell suspension in a culture medium. The concentration was adjusted to 2×10^6 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). Mitogenic stimulation was carried out as follows: 5×10^5 cells per well in a total volume of 50 μl were incubated with various doses of RGAP in the presence or absence (control) of the mitogens. The additions (50 ìl) are as follows: concanavalin A (Con A) at 4 μg/ml for T cell activation; and lipopolysacchride (LPS) at 10 μg/ml for B cell activation. After an incubation period of 72 hrs, the enzyme activity of the viable cells was measured by adding 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) to each well. After an additional incubation period of 4 hrs to allow for the dissolution of the MTT crystals, the absorption was measured at 540 nm using a microplate reader (Molecular Devices, Menlo Park, CA).

Assessment of NK cell cytotoxicity

The NK cell cytotoxicity was determined using a standard ^{51}Cr release assay.[16] Spleen cells from the male C57BL/6 mice were tested as effector cells and YAC-1 mouse lymphoma cells (ATCC, Rockville, MD) were used as the target cells labeled with a sodium ^{51}Cr-chromate solution (Amersham, UK). The NK cell assays were carried out in 96-well v-bottom plates at effector/target cell ratios 50:1 and 100:1 with 1×10^4 of the target cells in a final well volume of 200 μl for 6 hrs at 37°C in a 5% CO_2 humidified incubator. The specific release of ^{51}Cr by the YAC-1 target cells reflected the natural cytotoxic activity of the NK cells, and was calculated as follows: % cytotoxicity=[(E-S)/(T-S)]×100

where E is the activity of the ^{51}Cr released from the target cells in the presence of effector cells, S is the activity of ^{51}Cr released spontaneously from the target cells alone under identical conditions. T is the maximum activity of the ^{51}Cr released when all the target cells were destroyed.

Cytokine determination by ELISA

The splenocytes from the male B57BL/6 mice were cultured with or without RGAP for 16 hrs. The culture supernatants were collected and the IFN-γ concentration in the culture supernatants was determined using an ELISA kit according to the manufacturer's instructions (Endogen, Woburn, MA).

Statistical analysis

The data is represented as a mean ± S.E.M. The statistical difference between the groups was determined using a one-way analysis of variance (ANOVA) with a Dunnett's *t*-test. A P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

The cytolytic capacity of the NK cells was measured using a ^{51}Cr releasing assay at different E:T ratios. The *in vitro* effect of RGAP on the NK activity was examined at an E:T ratios of 50:1 and 100:1. Culturing the splenocytes with RGAP for 16 hrs resulted in the enhancement of NK cell activity, which was dose dependent. Two concentrations of RGAP (10 and 100 μg/ml) increased the NK cell activity by 141% and 239% at E:T ratio of 50:1, respectively. Treatment with 10 and 100 μg/ml of RGAP increased by 160% and 250% the NK cell activity at E:T ratio of 100:1 (Fig. 1). RGAP did not exert any cytotoxic activity on Yac-1 cells by themselves. This indicates that RGAP can activate the cytolytic activity of NK cells without affecting the cell viability.

In the next set of experiments, we determined the proliferative effects of RGAP on lymphocytes. Fig. 2 shows

the results of the MTT assays performed by stimulating the spleen cells with RGAP in the presence or absence of mitogens, ConA or LPS for 72 hours**.** RGAP stimulated slightly spleen cell proliferation at 100 μg/ml in the absence of mitogen, while RGAP exposure for 72 hours produced an increase of B cell and T cell proliferation in response to mitogens. It can be noted that the stimulating effect of RGAP was significantly stronger against the mitogenic response induced by LPS than against that induced by Con A. These data lead us to conclude that RGAP stimulated preferentially B lymphocytes.

The mechanism by which RGAP enhances the NK cell activity is not completely understood but might be due to an increase in the production of cytokines such as IFN-γ and TNF-α. Fig. 3 shows the results on the effect of RGAP on IFN-γ and TNF-α production from splenocytes.

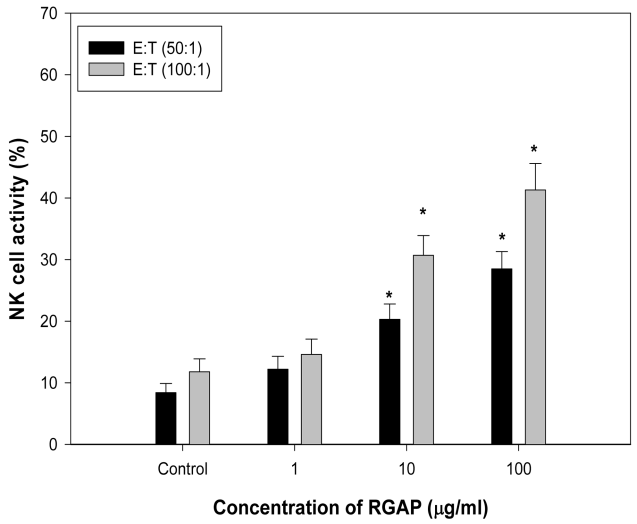

Fig. 1. *In vitro* effect of RGAP on the NK cell cytotoxicity. Splenocytes were cultured with various RGAP doses (1-l00 μg/ml) for 16 hrs. The NK cell cytotoxicity was examined at E:T ratios of 50:1 and 100:l. The data represents the mean ± SE of quadruplicate experiments. *: Significantly different from control (no treatment); $p<0.05$.

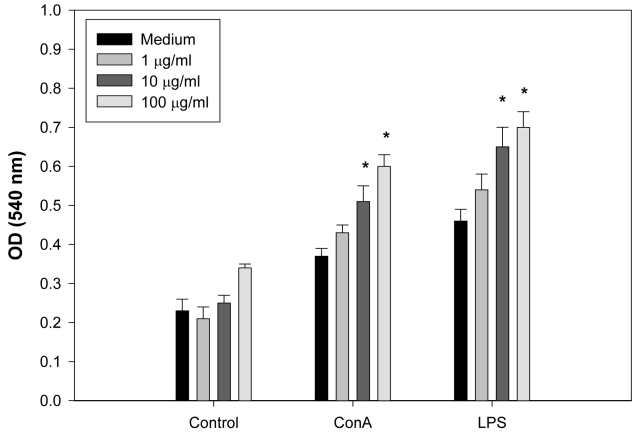

Fig. 2. *In vitro* effect of RGAP on the lymphocyte proliferation. The splenocytes were incubated with various RGAP concentrations (1-l00 μg/ml) in the presence or absence of Con A (4 μg/ml) and LPS (10 μg/ml) for 72 hrs, and the absorption was measured at 540 nm. The data is expressed as a mean ± SE of quadruplicates of a representative experiment. *: Significantly different from control (no treatment); $p<0.05$.

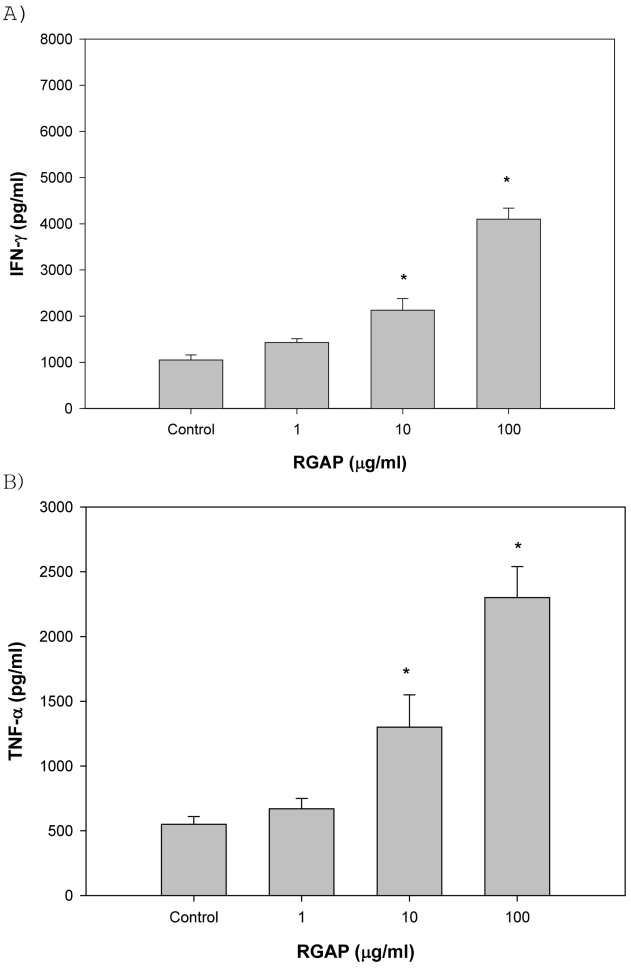

Fig. 3. *In vitro* effect of RGAP on IFN-γ (A) and TNF-α (B) production. The splenocytes were incubated with RGAP at different concentrations (1-100 μg/ml) for 16 hrs and the supernatants were analyzed for IFN-γ or TNF-α using an ELISA assay. The results are shown a mean ± SE of quintuplicates from a representative experiment. *: Significantly different from the control (no treatment); $p<0.05$.

The RGAP treatment resulted in a significant increase in IFN-γ and TNF-α production in a dose dependent manner, and was maximized at a concentration of l00 μg/ml. Overall, these findings clearly demonstrate that RGAP can induce the tumoricidal and secretory activity of NK cells.

NK cells comprise a heterogeneous population of large granular lymphocytes that are known to participate in both homeostatic and inflammatory host defense functions.[17] These functions include release of immunoreg~atory cytokines as well as antimicrobial activities. Many biological agents can induce rapid IFN production from the large granular lymphocytes (LGL), and it is the production of IFN that produces the self-activation of the NK activity in the LGL.[1] In addition, there is good experimental evidence that TNF-α can be produced by NK cells.[3] Based on these findings, the present data suggest that the increased tumoricidal activity by RGAP is mediated by the increased IFN-γ and TNF-α production.

The possibility that RGAP used to stimulate the NK cells contained a small amount of contaminants such as endotoxin cannot be ruled out. RGAP could be contaminated with endotoxins that have various immunostimulatory effects. Therefore, the RGAP was tested for the presence of endotoxin using the Limulus amoebocyte lysate assay in order to discount the possibility of any impurity problems that might interfere with the interpretation of the results. The RGAP contained the lower limit detectability (data not shown). Accordingly, the possibility of NK cell activation by contaminant was ruled out.

Immunological stimulation is attracting a great deal of attention as a major treatment modality in the management of cancer patients. Several studies have shown that a single administration of a biological response modifier (BRM) significantly enhanced the NK cell activity.[18,19] Accordingly, the augmentation of NK activity by different BRMs has been the focus of many investigations. Recently, we reported that RGAP has an immunostimulating effect on macrophages after *in vitro* exposure.[13]

In conclusion, RGAP is a potent immunomodulator that significantly enhances the NK cell activity *in vitro*. In addition, the dose used and the data presented is expected to expand the database on the effect of RGAP on the immune system. Furthermore, these results reaffirm that NK cells are the primary cells in the immune system for destroying tumor cells and that the induced tumoricidal activities of the NK cells may be mediated by IFN-γ and TNF-α. The high supplementary effect of RGAP and the absence of notable side effects make this material a promising immunotherapeutic agent for treating cancer patients.

REFERENCES

1. Djeu JY. Production of interferon by natural killer cells. Clin Immunol Allergy 3: 561-568 (1983)
2. Herberman RB, Callewaert DM. Mechanisms of cytotoxicity by NK cells. Academic Press, London (1985)
3. Ross ME, Caligiuri MA. Cytokine-induced apoptosis of human natural killer cells identifies a novel mechanism to regulate the innate immune response. Blood 89: 910-918 (1997)
4. Hersey P, Edwards A, McCarthy WH. Tumor-related changes in natural killer activity in melanoma patients. Influence of stage of disease, tumor thickness and age of patients. Int J Cancer 25: 187-194 (1980)
5. Wilmer LS, Bankhurst AD, Jumonville AJ, Saiki JH, Saiers JH, Dobemeck RC. Defects in natural cell activity and interferon response in human lung carcinoma and malignant melanoma. Cancer Res. 44: 852-856 (1984)
6. Ghoneum M, Ghonaim M, Namatalla G. NK cell activity in hepatocellular carcinoma and its relation to the etiology of the disease. J Clean Technol Environ. Toxicol Occup Med. 5: 267-284 (1996)
7. Lee KD, Huemer RP. Antitumoral activity of Panax ginseng extracts. Jpn J Pharmacol. 21: 299-301 (1971)
8. Yun TK, Yun YS, Han IW. Anticarcinogenic effect of long-term oral administration of red ginseng on newborn mice exposed to various chemical carcinogens. Cancer Detect Prev. 6: 515-525 (1983)
9. Yun TK, Choi SY. A case-control study of ginseng intake and cancer. Int J Epidemiol. 19: 871-875 (1990)
10. Park KM, Kim YS, Jeong TC, Joe CO, Shin HJ, Lee YH, Nam KY, Park JD. Nitric oxide is involved in the immunomodulating activities of acidic polysaccharide from Panax ginseng. Planta Med. 67: 122-126 (2001)
11. Shin HJ, Kim YS, Kwak YS, Song YB, Kim YS, Park JD. Enhancement of antitumor effects of paclitaxel (taxol) in combination with red ginseng acidic polysaccharide (RGAP). Planta Med. 70: 1033-1038 (2004)
12. Kim YS, Park KM, Shin HJ, Nam KY, Park JD. Anticancer activities of red ginseng acidic polysaccharide by activation of macrophages and natural killer cells. Yakhak Hoeji 46: 113-119 (2002)
13. Choi HS, Kim KH, Sohn E, Park JD, Kim BO, Moon EY, Rhee DK, Pyo S. Red ginseng acidic polysaccharide (RGAP) in combination with IFN-gamma results in enhanced macrophage function through activation of the NF-kappaB pathway. Biosci Biotechnol Biochem. 72: 1817-1825 (2008)
14. Chaplin MF. Monosaccharides. p. 1-40. In: Chaplin, M. F. and Kennedy, N. K.(ed), Carbohydrate Analysis. IRL Press,

Oxford (1994)
15. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth. 65: 55-63 (1983)
16. Kim KR, Son EW, Hee-Um S, Kim BO, Rhee DK, Pyo S. Immune alterations in mice exposed to the herbicide simazine. J Toxicol Environ Health A. 66: 1159-1173 (2003)
17. Reynolds CW, Wiltrout RH. Functions of the Natural Immune System. Plenum Press, New York (1989)
18. Herberman RB, Ortaldo JR. Natural killer cells: their role in defenses against disease. Science 214: 24-30 (1981)
19. Herberman RB, Thurman GB. Summary of approaches to the immunological monitoring of cancer patients treated with natural or recombinant interferon. J Biol Resp Modif. 2: 572-562 (1983)