White ginseng extract induces immunomodulatory effects via the MKK4-JNK pathway

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Abstract *Panax ginseng* Meyer (white ginseng) is a popular functional food and its biological effects on the human body have been noted for hundreds of years. In the present study, the underlying mechanisms responsible for the immunomodulatory effects of white ginseng extract (WGE) were investigated. WGE increased NO production via upregulation of iNOS expression levels. Mouse cytokine array results also revealed that the expression of 13 cytokines was elevated by WGE treatment in IFN- γ -primed macrophage cells. Although both MKK4-JNK and MEK-ERK signaling pathways were activated after treatment with WGE, only the MKK4-JNK signaling pathway appears to have any significant immunomodulatory significance. Oral administration of WGE for 28 days recovered cyclophosphamide (CY)-induced suppression of the immune system in mice via the MKK4-JNK pathway. Taken together, these findings suggest that the MKK4-JNK signaling pathway is a crucial mechanism of WGE-induced immunomodulation.

Keywords: cytokines, immunomodulation, JNK, MKK4, white ginseng extract

Introduction

The immune system serves as a crucial defense mechanism and is functionally divided into two sectors, non-specific (innate immunity) and specific defense (adaptive immunity) (1). In regards to innate immunity, macrophages, dendritic cells (DCs), and neutrophils play a role in recognizing pathogens and triggering the production of cytokines (2). Activated macrophages will respond by releasing cytokines and cytotoxic molecules including interleukin (IL) and nitric oxide (NO) (1,3). The production of cytokines in macrophages is known to be modulated by various signaling pathway. We previously reported that NO, tumor necrosis factor (TNF)- α and IL-6 are produced by TLR-2-mediated nuclear factor-kappa B (NF- κ B) activation (4). In addition, mitogen-activated protein kinase (MAPK) signaling pathways have been reported to upregulate cytokine induction via NF- κ B (2,5). These activators of cytokine production-related signaling pathways are therefore attractive targets for the development of novel immunomodulatory agents.

Ginseng refers to the root and rhizome of *Panax ginseng*, one of the most widely-known functional foods. Indeed, ginseng has been extensively used as a medicinal herb for more than 2,000 years (6). Among the several species of ginseng, Asian ginseng (*P. ginseng* Meyer) has been the most well-studied due to its biological

properties which include anti-cancer, anti-metabolic syndrome and anti-cardiovascular disease effects (7-9). Of particular note, accumulating evidence is indicating that ginseng also has immunomodulatory properties. (10,11). Recently, Kim *et al.* (12) reported that intake of red ginseng with vitamin C prevents viral infection-related lung inflammation via repression of the viral lytic cycle and enhancement of T- and NK cell activity. These effects consequently increased the survival rate of mice administered with red ginseng extract. Two previous studies have demonstrated the stimulatory effect on the immune system of poly- and oligo-saccharides from ginseng. In these studies, the isolated poly- and oligo-saccharides increased cytokine activity, as well as activation of MAPK and NF- κ B signaling pathways (4,13). Ginsenoside is reported to be a principle compound responsible for the beneficial effects of ginseng. Further *in vitro* and *in vivo* studies have highlighted various other immuno-stimulatory effects of ginsenosides (10,11,14). Wang *et al.* (11) reported an activated immune response resulting from treatment with ginsenoside Rg1 via upregulation of the NF- κ B and PI3K/Akt/mTOR pathways. Interestingly, the adjuvant activity of ginsenoside Re has been demonstrated using an inactivated rabies virus vaccine (RV), with ginsenoside Re amplifying the activity of RV (10).

In the present study, we sought to investigate the immunomodulatory effects of WGE in murine RAW 264.7 cells.

Material and Methods

Reagents Murine RAW 264.7 cells were purchased from the ATCC (ATCC® TIB-71™). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The MTS solution for cell cytotoxicity assays was purchased from Promega (Madison, WI, USA). Primary antibodies recognizing iNOS, phosphorylated MEK (Ser^{217/221}), total MEK, phosphorylated SEK1/MKK4 (MKK4, Ser²⁵⁷/Thr²⁶¹), phosphorylated p44/p42 MAPK (ERK1/2) (Thr²⁰²/Tyr²⁰⁴), phosphorylated SAPK/JNK (Thr¹⁸³/Tyr¹⁸⁵), phosphorylated c-Jun (Ser⁷³), and phosphorylated p90^{RSK} (Thr⁵⁷³) were purchased from Cell Signaling Technology (Danvers, MA, USA), while antibodies against total ERK, total p90^{RSK}, total MKK4, total JNK and total c-Jun were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Murine recombinant IFN- γ , LPS and polymyxin B were purchased from Sigma-Aldrich (St Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for CXCL10, TNF- α , and IL-6 were obtained from R&D Systems™ (Minneapolis, MN, USA).

Preparation of white ginseng extract (WGE) The roots of 6-year old *P. ginseng* Meyer were chopped into 1-5 mm pieces before the powder (5 g) was twice extracted with 100 mL of 80% (v/v) ethanol at 80°C for 3 h. The ethanol extraction products were then filtered with filter paper (Whatman No.2) and subsequently freeze-dried. The solid residue obtained was dissolved in sterile distilled water.

Cell culture The murine RAW 264.7 macrophages were maintained in DMEM containing 10% (v/v) FBS and 2 mM L-glutamine at 37°C in a 5% CO₂ incubator.

Cell cytotoxicity assay The cytotoxicity of the compounds was evaluated using Cell Titer 96 Aqueous Solution (Promega). Briefly, the cells were seeded in 96-well plates, before treatment for 24 h. MTS solution was added for 1 h to determine cell viability by evaluating the absorbance at 490 nm.

NO determination The production of nitrite was estimated using Griess reagent (1:1 of 0.1% N-1 naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid). 2x10⁵ cells/mL were seeded in 24-well plates and primed with IFN- γ (10 ng/mL) for 1 h, with each sample treated for 12 h. Equal volumes of Griess reagent and the cultured media were incubated at room temperature for 10 min. The absorbance was then measured at 540 nm.

Western blot RAW 264.7 macrophages were cultured in DMEM containing 10% (v/v) FBS at 70-80% confluency. The cells were then either left unprimed or primed with 10 ng/mL of IFN- γ for 1 h, before LPS (1 μ g/mL) was added for 4 h. The cells were then lysed with 1x Cell Lysis Buffer (Cell Signaling Technology) and protein concentration

was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Equal quantities of total protein were loaded onto a 10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA) for separation. The separated proteins were transferred to Immobilon P membranes (Millipore, Billerica, MA, USA). Non-specific proteins were blocked with 5% fat-free milk for 1 h, before the primary antibody was treated at 4°C overnight. Protein bands were detected with a chemiluminescence detection kit (GE Healthcare, Little Chalfont, UK) after hybridization with an HRP-conjugated secondary antibody (Cell Signaling).

Cytokine & phospho MAPK array Changes in cytokine and phospho-MAPK expression were analyzed with the Mouse Cytokine Array Panel A (R&D Systems™) and Human Phospho-MAPK Array (R&D Systems™), respectively. Briefly, the cells were cultured in DMEM containing 10% (v/v) FBS. After 1 h of IFN- γ priming, the cells were exposed to various concentrations of WGE. The process was carried out in accordance with the manufacturer's instructions.

Luminex multiplex analysis The quantitative cytokine assay was conducted using a Luminex multiplex analyser (Komabiotec, Seoul, Korea). After culturing in 24-well plates up to 70-80% confluency, 10 ng/mL of IFN- γ was treated for priming. The cells were then exposed to various concentrations of WGE for 12 h. The cultured media was analyzed for the quantitative estimation.

Animals BALB/C female mice (8-week-old) were obtained from Koatec Animal Inc. (Pyeongtaek, Korea), and maintained under controlled conditions (temperature: 20±2°C, humidity: 40–60%, light/dark cycle: 12 h).

Cyclophosphamide-induced immunosuppression in mice The mice were divided into 5 groups ($n=6$): Group 1 (saline-treated control, N.C.); Group 2 (Cyclophosphamide alone-treated group, CY) administered with saline for a period of the experiment; Groups 3-4 (WGE+cyclophosphamide) received WGE (200 or 400 mg/kg/day, p.o.) daily for 28 days; Group 5 (cold-fx+cyclophosphamide) was given cold-fx 200 mg/kg/day, p.o. daily for 28 days. Groups 2-5 were injected with cyclophosphamide (150 mg/kg/day, i.p.) on the 8th, 9th, and 10th day after 1 h of respective treatment. The mice were sacrificed at 28 days, and relative organ weights (organ weight/body weight) of the thymus and spleen were determined for each individual.

Immunohistochemical staining Immunohistochemical staining was analyzed by Abion CRO (Seoul, Korea). Briefly, the spleen and thymus were fixed in 10% formalin, and the tissues were dehydrated using a graded ethanol series. In accordance with general protocols, the tissue was processed for embedding in paraffin wax. To exclude endogenous peroxidase activity, the sections were incubated in 0.3% H₂O₂ for 15 min, and then each primary antibody was treated at

1:200 dilution factors for 1 h. The detection system visualized anti-mouse antibodies (K4001; DAKO, Glostrup, Denmark) and was applied according to the manufacturers' instructions. Slides were stained with liquid diaminobenzidinetetrahydrochloride (DAB+), a high-sensitivity substrate-chromogen system (K3468; DAKO). The images on the slides were visualized with an Olympus BX40 light microscope.

The chemical compositions and chromatographic conditions The content of ginsenosides was estimated using UHPLC. The analytical instrument was performed by a ChromasterUltra Rs UHPLC system (Hitachi-High Technologies, Tokyo, Japan) composed of binary pump, autosampler, UV-vis detector, and column oven. The chromatographic separation was accomplished on an ACQUITY BEH C18 column (2.1 mm×50 mm, 1.7 μ m; Waters, Milford, MA, USA). The column temperature was 45°C. The binary gradient elution system was consisted of 0.001% phosphoric acid in water (A) and in acetonitrile (B). The separation was performed using following program: 15% B (0-0.5 min), 30% B (14.5 min), 32% B (15.5 min), 38% B (18.5 min), 41% B (22 min), 55% B (25-29 min), 70% B (33 min), 90% B (35-37 min), 15% B (38 min), 15% B (41 min). The flow rate set at 0.6 mL/min and the sample injection volume was 3.0 μ L. The ginsenosides were detected at 203 nm.

Statistical analysis All experiments were performed at least three times. Data are expressed as mean and SD values, and Student's *t*-test and Scheff's test were used for single statistical comparisons and the intergroup differences, respectively. The SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA) was used to analyze the data. A probability cutoff of *p*<0.05 was used as the criterion for statistical significance.

Results and Discussion

White ginseng extract induces NO production and iNOS expression in IFN- γ -primed RAW 264.7 macrophages Increased NO production by iNOS is one of the major mechanisms of host defense against bacterial infection (15). We therefore analyzed iNOS expression levels after WGE treatment in IFN- γ -primed RAW 264.7 cells. As seen in Fig. 1A, WGE did not show any cytotoxic effects with up to 500 μ g/mL concentrations within 24 h. WGE treatment was observed to upregulate iNOS expression levels (Fig. 1B), with the WGE 125 μ g/mL group showing an almost 2-fold higher expression of iNOS than the control IFN- γ -primed group. Similar to the iNOS results, NO production levels were significantly increased by WGE treatment. Over 5-fold increases in NO levels were observed for 500 μ g/mL treatments of WGE in comparison to the IFN- γ -primed group (Fig. 1C). Although it is already reported the anti-inflammatory potential and anti-phagocytic activity of total saponins and acidic polysaccharide from Korean red ginseng using LPS-induced inflammation model (16)

and intracellular *Brucella* infection model (17), respectively, current study used normal condition model to confirm the immunomodulation activity of the ginseng. These contrasting results of products from *Panax ginseng* Meyer are concluded to the maintaining function of immune homeostasis of *Panax ginseng* Meyer.

White ginseng extract upregulates cytokine production in IFN- γ -primed RAW 264.7 macrophages Proinflammatory cytokines, such as those from the TNF- α and IL families, play an important role in immune and inflammatory responses (18). In particular, increment in proinflammatory cytokine production is a representative host defense reaction toward pathogenic bacteria and parasites (3). We assessed the effect of WGE on cytokine expression levels in IFN- γ -primed RAW 264.7 macrophages. The cytokine levels in the culture supernatant were evaluated after 12 h of WGE treatment. While IFN- γ treatment did not cause any remarkable changes in cytokine expression, treatment of WGE in IFN- γ -primed RAW 264.7 macrophages caused the induction of 13 different cytokines including CXCL10, CXCL11, TNF- α , IL-1 α and IL-23 (Fig. 2A). LPS was used as positive control for the experiment, and changes in cytokine levels for the WGE treated group exhibited similar results with the LPS treatment group (Fig. 2A). For the quantitative analysis, we analyzed the representative cytokine production levels using Luminex multiplex analysis as described in the Materials and Methods. LPS treatment significantly increased CXCL10 (Fig. 2B), IL-6 (Fig. 2C) and TNF- α (Fig. 2D) expression levels and these cytokines were also elevated by treatment with WGE (Fig. 2B-2D).

MEK-ERK-p90^{RSK} and MKK4-JNK-c-Jun signaling pathways are upregulated after treatment with white ginseng extract As the production of immune-related cytokines is closely related to MAPK signaling pathway activity (19,20), we screened the activation of 26 different MAPKs after treatment with WGE in IFN- γ -primed RAW 264.7 cells. JNK1, JNK2 and ERK2 were found to be highly phosphorylated by WGE treatment (Fig. 3A). Although the phosphorylation of CREB and p70^{S6K} were also increased, we assumed these increments are caused by activation of JNK and ERK (21,22). Next, we sought to verify whether WGE affects ERK or JNK signaling pathways. Phosphorylated ERK was increased by WGE treatment and furthermore, the known upstream and downstream signaling factors for ERK, MEK and p90^{RSK}, respectively (23) were phosphorylated by WGE (Fig. 3B). Phosphorylated-MKK4 and -c-Jun were assessed as the upstream and downstream factors of JNK, respectively. The MKK4-JNK-c-Jun signaling pathway was enhanced by WGE treatment in a dose-dependent manner (Fig. 3C). These findings indicate that production of immunostimulating cytokines by WGE may result from MAPK activation.

Activation of JNK signaling pathway, but not ERK signaling, is primarily responsible for the immuno-stimulatory activity of WGE We next investigated whether the ERK or JNK signaling pathways

Fig. 1. Effect of WGE on iNOS expression and NO production in IFN- γ -primed macrophage cells under non-cytotoxic concentration. (A) Cytotoxicity of WGE was determined using the MTS assay. The data are presented as means $\pm$ SD of three independent experiments. The asterisk (*) indicates a significant ($p < 0.01$) decrease in cytotoxicity with untreated control. (B) Activation effect of WGE on the expression level of iNOS. The protein level of iNOS was measured using Western blot assay. (C) NO producing effect of WGE. The detailed procedure was described in Material and Methods. In brief, WGE was treated to the cells for 12 h and the NO level was measured. The NO level was estimated using Griess reagent. (D) Major ginsenoside contents in WGE. The ginsenoside contents was analyzed using UHPLC. Detail chromatographic conditions were represented in "Material and Methods" section. The data are representative of three independent experiments that gave similar results.

affect WGE-induced immuno-stimulatory cytokine production using pharmacological inhibitors of ERK and JNK, PD98059 and SP600125, respectively. We pre-treated PD98059 or SP600125 for 1 h prior to treatment with WGE as described in the Material and Methods. Interestingly, the expression of iNOS was only attenuated by JNK inhibitor treatment (SP600125) but not the ERK inhibitor (PD98059) (Fig. 4A). Similarly, the production of NO was decreased by SP600125 but not PD98059 (Fig. 4B). We next quantitatively analyzed the production of IL-6 and TNF- α . Although IL-6 levels were also reduced by PD98059 treatment, SP600125 suppressed WGE-induced IL-6 (Fig. 4C) and TNF- α (Fig. 4D) protein levels concurrently. Overall, although

MEK-ERK-p90^{RSK} and MKK4-JNK-c-Jun signaling pathways were activated by WGE treatment, only WGE-induced MKK4-JNK-c-Jun signaling was demonstrated to be responsible for the immuno-stimulatory effects of WGE in IFN- γ -primed RAW 264.7 macrophages.

White ginseng extract exhibits immuno-stimulatory effects in a mouse model

To confirm the immuno-stimulatory effect of WGE in more physiological conditions, we used a cyclophosphamide (CY)-induced immunosuppressed mouse model with BALB/C female mice, as described in the Material and Methods. 200 or 400 mg/kg of WGE was orally administered for 28 days (Fig. 5A) and NK cell activity,

Fig. 2. Effect of WGE on cytokine production in IFN- γ -primed macrophage cells. (A) 26 cytokines expression were analyzed using cytokine array. Total procedure was explained in Material and Methods. WGE was treated to the cells for 12 h and the cytokines was evaluated in supernatant. (B-D) Quantitative analysis of representative cytokines, (B) CXCL10, (C) IL-6 and (D) TNF- α . The level of each cytokines was analyzed using Luminex multiplex analyser (Komabiotech). The data are representative of three independent experiments that gave similar results.

as well as the proliferation of T and B lymphocytes were analyzed. NK cell activity in the CY-treated group was dramatically decreased and intake of WGE recovered CY-suppressed NK cell activity (Fig. 5B). NK cell activity was measured using NK cell-sensitive YAC-1 cells (24,25). Administration of cold-fx was used as a positive control (26,27). Although, we could not find a significant difference using 12.5:1 (NK cell : YAC-1), the ratio 25:1 and 50:1 represented increased NK cell activity in Fig. 5B. Next, the proliferation of T and B lymphocytes was measured. Similar with the results for NK cell activity, the proliferation of T and B lymphocytes was alleviated by i.p. injection of

CY and WGE revoked CY-suppressed proliferation of T and B lymphocytes (Fig. 5C).

White ginseng extract induces cytokine expression in the spleen and thymus Next, we investigated the effect of WGE application on the expression of TNF- α and IL-6 in spleen and thymus using immunohistochemical staining. In Fig. 6A and 6B, the expression levels of TNF- α and IL-6 were significantly increased by WGE intake. The integrity of the immune system is closely linked with severity of various human diseases including cold, asthma, eczema, and even

Fig. 3. Effect of WGE treatment on MAPK phosphorylation in IFN- γ -primed macrophage cells. (A) 26 different MAPKs was screened using phospho-MAPK array. The cells were treated with WGE for 3 h. And the phospho-MAPKs level was assessed. The whole procedure followed by manufacturer's instructions. (B and C) The effect of WGE on (B) MEK-ERK-p90^{RSK} and (C) MKK4-JNK-c-Jun signaling pathway was estimated using Western blot assay. Briefly, the cells were primed with IFN- γ for 1 h and WGE was treated to the cells for 3 h. The data are representative of three independent experiments that gave similar results.

cancer and metabolic disorders (28,29), A considerable amount of research has thus focused on the identification and development of agents that can modulate the immune system (1,11,12,24). Ginseng has been renowned as an immune-boosting herb for thousands of years (6). However, the specific underlying mechanisms of such action by ginseng have yet to be clearly understood.

Using a cytokine array, we determined that 13 cytokines are upregulated by WGE treatment in IFN- γ -primed RAW 264.7 cells. Additionally, this immune-boosting activity of WGE was supported by evidence from a CY-induced immunosuppressed mouse model. In this *in vivo* study, oral intake of WGE increased NK cell activity and the proliferation of B- and T-lymphocytes. We analyzed the weight of another immune-related organ, the thymus. Although we could not find a statistical correlation between CY- and CY plus WGE-treated groups in terms of relative thymus weight, a tendency toward recovery was noted. To eliminate the effect of decreased body weight due to immunosuppression by CY treatment on spleen and

thymus weight, we compared each organ weight versus the total body weight.

To elucidate the underlying mechanisms of WGE-associated immuno-stimulatory activity, we evaluated the effect of WGE on the phosphorylation levels of 26 kinases (Fig. 3A). We positively identified upregulated JNK, CREB, p70^{S6K} and ERK2 phosphorylation. As increases in phosphorylated CREB or p70^{S6K} by WGE might be due to the effect of activated JNK or ERK (21,22), we only investigated the JNK and ERK signaling pathways. Interestingly, even though WGE activated the MEK-ERK-p90^{RSK} signaling pathway, PD98059, a pharmaceutical inhibitor of ERK did not affect WGE-induced NO production, or iNOS expression (Fig. 4A and 4B). However, IL-6 production was diminished by PD98059 treatment (Fig. 4C). We assume that this reduction might due to the relationship between MEK-ERK-p90^{RSK} pathway and IL-6 production. A previous study reported the effect of PD98059 on LPS-induced IL-6 production, and suggested that production of IL-6 is mediated by ERK1/2 activation

Fig. 4. Role of the JNK signaling pathway on immune-stimulatory activity of WGE. The cells were pretreated with PD98059 (an ERK inhibitor) or SP600125 (a JNK inhibitor) and then WGE was treated to the cells for 12 h. (A) The protein level of iNOS was estimated using Western blot assay. (B) Amount of the NO was estimated using Griess reagent in Material and Methods. The level of (C) IL-6 and (D) TNF- α was analyzed using Luminex multiplex analyser (Komabiotek). The data are representative of three independent experiments that gave similar results.

Fig. 5. Immuno-stimulatory activity of WGE in animal model. (A) Total period of animal study. WGE was orally administered for 28 days. And Group 2 (CY alone-treated group), Group 3-4 (WGE + CY group) and group 5 (cold-fx + CY group) were injected with CY (150 mg/kg/day, i.p.) on the 8th, 9th, and 10th day after 1 h of respective treatment. (B) NK cell activity was measured using NK cell-sensitive YAC-1 cells. We analyzed NK cell activity using 12.5:1 (NK cell : YAC-1), 25:1 and 50:1. (C) The proliferation of T and B lymphocytes was measured for evaluating Immuno-stimulatory activity of WGE.

(30). To support our notion, we analyzed MKK4 and JNK phosphorylation in the spleen, and phosphorylated MKK4 and JNK was observed in the WGE-administered group.

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Disclosure The authors declare no conflict of interest.

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