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Immunostimulating and Antimetastatic Effects of Polysaccharides Purified from Ginseng Berry

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Abstract: Ginseng root has been used in traditional oriental medicine for the enhancement of immune system function. The immunostimulatory effects of ginseng berry polysaccharides, however, remain unclear. Effects of polysaccharides from ginseng berry on the activation of natural killer (NK) cells and inhibition of tumors are reported. A crude polysaccharide was isolated from ginseng berry as a ginseng berry polysaccharide portion (GBPP) and was further fractionated using gel filtration chromatography to obtain the three polysaccharide fractions GBPP-I, -II and -III. GBPP-I consisted of mainly galactose (46.9%) and arabinose (27.5%). GBPP-I showed a high dose-dependent anticomplementary activity. Stimulation of murine peritoneal macrophages by GBPP-I showed the greatest enhancement of interleukin (IL)-6 and IL-12 and tumor necrosis factor (TNF)-α production. In addition, an *ex vivo* assay of natural killer (NK) cell activity showed that oral (*p.o.*) administration of GBPP-I significantly increased NK cell cytotoxicity in YAC-1 tumor cells and production of granzyme B. Prophylactic intravenous (*i.v.*) and *p.o.* administration

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of GBPP-I significantly and dose-dependently inhibited lung metastatic activity in B16BL6 melanoma cells. Depletion of NK cells after injection of rabbit anti-asialo GM1 partially abolished the inhibitory effect of GBPP-I on lung metastasis, indicating that NK cells play an important role in anticancer effects. GBPP-I exerts a strong immune-enhancing activity and can prevent cancer metastasis through activation of NK cells and other immune-related cells.

Keywords: Panax Ginseng Berry; Polysaccharide; Antimetastatic Activity; Macrophage; Natural Killer Cell; Asialo GM1.

Introduction

Cancer is one of the biggest causes of death in the world despite development of numerous treatments and medications. The main cause of death from cancer is damage to the immune system during tumor therapy. Therefore, it is important to study new anticancer substances in view of improving immunity without harming the host (Ooi and Liu, 2000). Over the past few decades, polysaccharides and polysaccharide protein complexes have been isolated from natural products. Recently, a variety of polysaccharide biological activities (such as immunomodulatory and antitumor effects) have attracted attention in biochemical and medical fields (Wasser, 2002). Several studies have shown that polysaccharides isolated from natural products can enhance the immune response by promoting the antitumor activities of macrophages and natural killer cells and can also enhance adaptive immune responses (Ooi and Liu, 2000; Yim *et al.*, 2005; Schepetkin and Quinn, 2006). In addition, some cytokines, such as TNF-α, IL-6 and IL-12 from macrophages, increase NK cell responses, and can induce activation of adaptive immunity in part through stimulation of IFN-γ production in NK cells (Yoon *et al.*, 2008).

Panax ginseng C.A. Meyer has been traditionally used as an important herbal medicine in Asia. Ginseng importance lies in its multiple pharmacological functions, such as an anticancer activity, and antistress, antifatigue, anti-oxidant and anti-aging effects (Attele *et al.*, 1999). The major ginsenoside compounds of *P. ginseng* are distributed in many parts of the ginseng plant, including the root, berry and leaf. Different parts of the plant contain distinct ginsenoside profiles (Attele *et al.*, 1999) and may have different pharmacological activities. Most *P. ginseng* research has focused on study of the active ginsenoside ingredients in the root. Recent reports have revealed that a polysaccharide extracted from the ginseng root has a considerable immunostimulating activity, including immune modulation (Choi *et al.*, 2008), inhibition of tumors (Shin *et al.*, 2004) and inhibition of pathogenic bacteria (Lee *et al.*, 2006). In addition to ginseng root, other parts of the ginseng plant, such as the berry and leaf, contain considerable amounts of polysaccharides (Sun, 2011). Studies on polysaccharides from ginseng berry, however, are limited. In this study, a pectic polysaccharide from ginseng berry was isolated, purified and evaluated for immune activation effects, especially NK cell activation and the anticancer immune response.

Materials and Methods

Materials and Animals

Ginseng berries were collected in Keumsan, Chungnam Province, Korea (2016). Specific pathogen-free (SPF) female BALB/c mice (6–8 weeks old) were purchased from Saeronbio Inc. (Gyeonggi, Korea). Mice were maintained in a clean rack under a 12/12 h light/dark cycle at Kyonggi University. Water and a pellet diet were supplied *ad libitum*. All animal experiments were carried out according to the instructions of the Ethics Committee for Use of Experimental Animals at Kyonggi University (2017-005) and were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996).

Isolation and Purification of a Polysaccharide from Ginseng Berry

Material for the study, including a ginseng berry polysaccharide extract (GBPE) and the ginseng berry polysaccharide portion (GBPP) were obtained from AmorePacific R&D (Gyeonggi-do, Korea). Preparation methods are briefly described as follows: Korean ginseng berries were harvested and seeds were removed. The berry flesh was first refluxed with 90% ethanol for 5 h, and then the remaining residues were extracted using water at 100°C for 5 h to obtain a water extract of ginseng berry. The supernatant of the extracted mixture was collected. Following addition of 4 volumes of 95% ethanol to the supernatant, crude berry polysaccharides were precipitated, and then lyophilized to obtain a ginseng berry polysaccharide extract (GBPE), which was dissolved in water and dialyzed using a molecular weight cut-off of 20 kDa. The dialyzed solution was then lyophilized to obtain the ginseng berry crude polysaccharide portion (GBPP), which was subjected to Sephadex G-75 gel permeation column (2.5 × 90 cm) chromatography, and the three major polysaccharide fractions GBPP-I, GBPP-II and GBPP-III were obtained. Each fraction was lyophilized after desalting by dialysis (molecular weight cut-off of 12–14 kDa, Spectrum Laboratories Inc.).

Analysis of Sugar Components and Molecular Weight

Total carbohydrate contents were determined using the phenol-sulfuric acid method (DuBois *et al.*, 1956) with galactose as a reference. The amount of uronic acid was measured following the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1937) using galacturonic acid as a reference. The 2-*keto*-3-deoxy-D-manno-octulosonic acid (KDO) content was determined following a modified thiobarbituric acid (TBA) method (Karkhanis *et al.*, 1978) using KDO as a reference. The protein content was determined following the Bradford method (Bradford, 1976) using bovine serum albumin as a reference. The monosaccharide composition of polysaccharides was analyzed following the partially modified alditol acetate method of Jones and Albersheim (Jones and Albersheim, 1972) using gas chromatography (GC) equipment (6000 series, Young-Lin Co., Anyang, Korea) equipped with an SP-2380 capillary column (0.2 μm × 0.25 mm × 30 m;

Supelco, Bellefonte, PA, USA) and a flame ionization detector (FID). The temperature program of the GC was 60°C for 1 min, 60 → 220°C (30°C/min), 220°C for 12 min, 220 → 250°C (8°C/min) and 250°C for 15 min. The molar ratio of monosaccharides was calculated from peak areas and response factors. The polysaccharide molecular weight was confirmed based on high performance size-exclusion chromatography (HPSEC) using an Agilent 1260 Infinity LC system (Agilent Technologies Co., Ltd., Palo Alto, CA, USA) equipped with a Superdex™ 75 GL column (GE Healthcare, Anaheim, CA, USA) and a refractive index detector (Agilent 1200 series). A total of 20 μL of a polysaccharide solution was analyzed using an isocratic mobile phase (50 mM ammonium formate buffer, pH 5.5) at a flow rate of 0.5 mL/min at 25°C. The molecular weights of polysaccharides were calculated from the calibration curve generated for standard pullulans (P-800, 400, 200, 100, 50, 20, 10, and 5; Showa Denko, Co. Ltd., Tokyo, Japan).

Anticomplementary Activity

Complement activation was measured following the method of Mayer with some modification (Mayer, 1964). Fresh normal human sera (NHS) were prepared from healthy adult volunteers from The Department of Food Science and Biotechnology, Kyonggi University, Korea. Freshly drawn whole blood was centrifuged at 800 × *g* for 10 min after standing for 30 min. Supernatant NHS was pooled, divided into 1 mL aliquots and preserved at −70°C before used for an assay. Frozen preparations were immediately thawed before use. Gelatin-Veronal buffered saline (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+} (GVB^{2+}) were prepared. Polysaccharides (50 μL) in ice water were incubated with NHS (50 μL) and GVB^{2+} (50 μL). Mixtures were incubated at 37°C for 30 min, and 350 μL of GVB^{2+} was added. IgM-hemolysin-sensitized sheep erythrocyte (EA) cells adjusted to 1×10^8 cells/mL (250 μL) were added to the mixtures, diluted serially (10 ∼ 160 fold), then incubated at 37°C for 60 min. After addition of 2.5 mL of phosphate buffered saline (PBS, pH 7.4), centrifugation followed at 700 × *g* for 10 min. The absorbance of supernatants was detected at 412 nm. NHS was separately incubated with both water and GVB^{2+} to provide a control. The anticomplementary activity of the polysaccharide was expressed as a percentage inhibition of 50% of total complement hemolysis ($ITCH_{50}$) of the control. $ITCH_{50}$ (%) = [(TCH_{50} of control — TCH_{50} treated with sample)/TCH_{50} of control] ×100.

Cell Cultures

Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and fungizone (amphotericin B) were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). A lung metastatic subline of B16-BL6 melanoma cells was maintained in DMEM supplemented with 5% FBS, penicillin/streptomycin, a vitamin solution, sodium pyruvate, non-essential amino acids and l-glutamine. Mouse peritoneal macrophages and an NK-sensitive mouse lymphoma cell line of YAC-1 cells were maintained in RPMI-1640

medium supplemented with 5% FBS, penicillin/streptomycin and L-glutamine. Cells were cultured at 37°C under humidified air containing 5% CO_2.

Macrophage Cytotoxicity and Cytokine Production

Peritoneal macrophages were collected from thioglycollate (TG)-treated BALB/c mice (Saiki *et al.*, 1988) and seeded at a density of 1×10^6 cells/well in complete RPMI-1640 medium for 2 h in a 5% humidified CO_2 incubator. Non-adherent cells were removed using PBS and peritoneal macrophages were further treated with polysaccharides fractions for 24 h. The cytotoxic effects of fractions were evaluated using a conventional WST assay with a Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA). The viability of treated cells was expressed as a percentage of the value of negative control cells treated with the medium alone. Supernatants were used for detection of cytokines. Levels of interleukin (IL)-6, IL-12, and tumor necrosis factor (TNF)-α were measured in the culture supernatant using sandwich enzyme-linked immunosorbent assay (ELISA) sets (BD Bioscience, San Diego, CA, USA) according to manufacturer protocols.

Assays for NK-Mediated Tumor Cytotoxicity and Granzyme After Oral Administration

To analyze the effects of GBPPs on activation of NK-cells in mouse splenocyte samples, GBPP-1 was orally (*p.o.*) administered to BALB/c mice ($n = 8$ per group) daily for 15 days, after which mice were sacrificed and splenocytes were harvested and prepared in a serum free medium (Hybridoma SFM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). NK cells were isolated using an NK Cell Isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer instructions. NK-cells were co-cultured with YAC-1 cells (1×10^5 cells/well) to obtain E/T ratios of 100:1, 50:1 and 25:1 in sterilized, U-bottomed 96-well plates. The E/T ratio indicates the ratio of effector cells (splenocytes) to target cells (YAC-1 cells). After 6 h, the supernatant was collected following centrifugation ($900 \times g$, 5 min) and combined with lactate dehydrogenase (LDH) reagent (Promega Co., Madison, WI, USA) in a new 96-well plate. NK cell-mediated tumor cytotoxicity was determined based on measurement of LDH levels in culture supernatants. The percentage of NK cell cytotoxicity was calculated using the following formula: Cytotoxicity (%) = [(absorbance value of experimental group — absorbance value of control group)/(absorbance value of untreated group — absorbance value of control group)] ×100. Also, levels of granzyme B released from activated NK cells were measured using appropriate ELISA sets (BD Bioscience).

Depletion of NK Cells In Vivo

Depletion of NK cells *in vivo* was performed according to a method previously described (Kasai *et al.*, 1981). Mice were injected intraperitoneally (*i.p.*) with 500 μL/mouse of rabbit anti-asialo GM1 serum (Wako Pure Chemicals Industries, Ltd., Japan) 2 days before tumor

inoculation. Procedures after anti-asialo treatment were the same as for the experimental lung metastasis experiment.

Experimental Lung Metastasis

GBPP-I inhibition effects on lung metastasis were measured after mice were treated with GBPP-I both orally (*p.o.*) (daily for 15 days) and intravenously (*i.v.*) (twice, 3 days and 1 day prior to *i.v.* tumor cell inoculation). Experimental lung metastasis was assessed based on *i.v.* inoculation with B16BL6 melanoma cells (2.7×10^4 cells per mouse) into syngeneic BALB/c mice 14 days after tumor inoculation followed by sacrifice. Lungs were fixed in Bouin's solution (Choon *et al.*, 1994) and lung tumor colonies were counted under a dissecting microscope.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS), Version 12.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as a mean ± standard deviation (SD) of three independent experiments performed in triplicate. Statistical significance between the two groups was determined using Student's *t*-test.

Table 1. Chemical Properties and Sugar Components of GBPP Subfractions Purified from Ginseng Berries

	GBPP-I	GBPP-II	GBPP-III
Yield from GBPP (%)	22.5	17.85	10.65
Chemical Composition (%)			
Neutral Sugar	89.1 ± 2.6	61.3 ± 3.6	52.9 ± 4.0
Uronic Acid	10.4 ± 0.8	16.1 ± 0.2	13.0 ± 0.4
Protein	0.2 ± 0.3	10.9 ± 0.2	10.7 ± 1.5
KDO-Liked Material	0.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
Phenolic Compound	0.2 ± 0.3	10.7 ± 1.5	22.1 ± 0.4
Component Sugar			(Mole %)
2-Methyl Fucose	—	0.5 ± 0.2	—
Rhamnose	6.7 ± 0.1	11.3 ± 0.1	5.5 ± 0.1
Fucose	—	0.5 ± 0.0	0.2 ± 0.1
2-Methyl Xylose	—	0.4 ± 0.1	—
Arabinose	27.5 ± 1.0	14.2 ± 0.2	6.9 ± 0.0
Xylose	0.4 ± 0.0	1.8 ± 0.1	7.8 ± 0.2
Apiose	—	—	—
Aceric Acid	—	0.2 ± 0.1	—
Mannose	2.3 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Galactose	46.9 ± 3.5	26.6 ± 0.2	23.4 ± 0.2
Glucose	5.4 ± 4.3	4.8 ± 0.1	8.1 ± 0.2
Galacturonic Acid	10.4 ± 0.8	16.1 ± 0.2	13.0 ± 0.4

A p-value of < 0.05 was considered as statistically significant. Differences among groups were evaluated following a one-way analysis of variance (ANOVA) and Duncan's multiple range test where $p < 0.05$ was considered statistically significant.

Results

Chemical Characteristics of GBPP Subfractions Purified from Ginseng Berry

GBPP was subjected to a Sephadex G-100 column for characterization of immune-stimulatory polysaccharides in berry and the crude polysaccharide fraction. Based on different analyses of each fraction for identification of neutral sugars, uronic acids, proteins and KDO-like materials, crude polysaccharides appeared as three major peaks (Fig. 1A). These fractions were successively separated into GBPP-I (76 kDa) (the first peak, Fig. 1B),

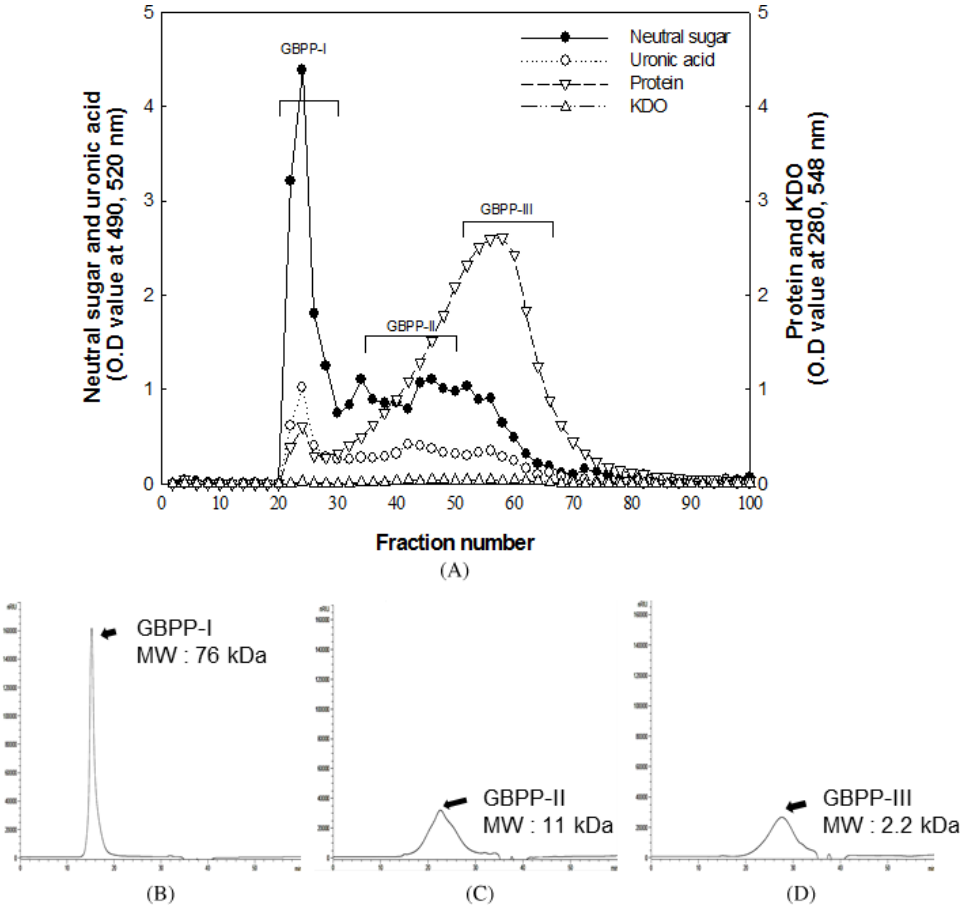


Figure 1. Elution profile for GBPP after gel permeation chromatography using Sephadex G-100 (A) and the molecular weights of GBPP-I (B), GBPP-II (C) and GBPP-III (D).

GBPP -II (11 kDa) (the middle peak, Fig. 1C) and GBPP-III (2.2 kDa) (the last peak, Fig. 1D). Each fraction was lyophilized following dialysis to obtain dry materials (yields from GBPP were 22.50% for GBPP-I, 17.85% for GBPP-II and 10.65% for GBPP-III, relative to the dry base) (Table 1). Based on a monosaccharide compositional analysis of the three fractions (Table 1), GBPP-I consisted of 89.1% neutral sugars (46.9% galactose, 27.5%, arabinose, and 10.4% galacturonic acid). In contrast, GBPP-II was composed of 61.3% neutral sugars (11.3%, rhamnose, 14.2% arabinose, 26.6% galactose, and 16.1% galacturonic acid). GBPP-II also contained considerable amounts of a Kdo-like material (1.0%) and unusual sugars, such as 2-methylfucose (0.5%), 2-methylxylose (0.4%) and aceric acid (0.2%). Together with large amounts of galacturonic acid, rhamnose, and arabinose in GBPP-II, Kdo-like materials and unusual sugars are known indicators of the presence of the RG-II polysaccharide (Shin *et al.*, 1997), indicating that GBPP-II was composed of RG-II and other polysaccharides. Finally, monosaccharide composition analysis revealed that the smallest molecular weight (MW) fraction, GBPP-III, consisted of eight different sugars of different compositions. Based on the finding that GBPP-III was enriched with phenolic compounds and sugars, GBPP-III likely contained hydrolyzed pectic polysaccharides during ripening of ginseng berry.

Anticomplementary Activities of GBPP Subfractions

The anticomplementary activities of the purified polysaccharides GBPP-I, GBPP-II and GBPP-III were examined in NHS (Fig. 2). Anticomplementary activity increased with an

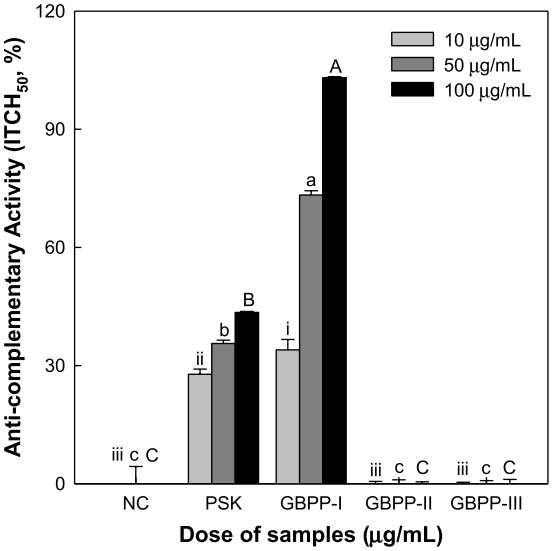


Figure 2. Anticomplementary activities of purified polysaccharide fractions (GBPP-I, GBPP-II, and GBPP-III) from ginseng berry. Values are expressed as a mean ± SD of three independent experiments performed in triplicate. $^{A-C,a-c,i-iii}$Bars not sharing the same superscript are significantly different from each other ($p < 0.05$).

increasing concentration of GBPP-I, reaching maximal activation at 100 μg/mL. GBPP-I exhibiting a 2.4-fold higher anticomplementary activity than PSK, a known immuno-active polysaccharide from *Coriolus versicolor* at 100 μg/mL (Tsukagoshi *et al.*, 1974). GBPP-II and GBPP-III, however, did not exhibit anticomplementary activities, even at a high concentration of 100 μg/mL.

Cytotoxicity of GBPP-I in Normal and Tumor Cells

The cytotoxic effects of GBPP-I were examined in murine macrophage cells and tumor cells. Macrophage and B16BL6 melanoma cells were treated with GBPP-I concentrations up to 100 μg/mL. GBPP-I treatment did not affect cell viability compared with a negative control (Figs. 3A and 3B).

Enhanced Cytokine Production for GBPP Subfractions

To investigate macrophage activation by GBPP subfractions (I–III), levels of the three cytokines IL-6, IL-12 and TNF-α that are produced by activated peritoneal macrophages were measured. GBPP-I showed the highest cytokine production, especially at lower dosages of 0.8–6.3 μg/mL (Figs. 3C–3E). Treatment with GBPP-I at concentrations of 100 μg/mL increased the level of IL-6 by 90-fold, whereas the level of IL-12 increased by

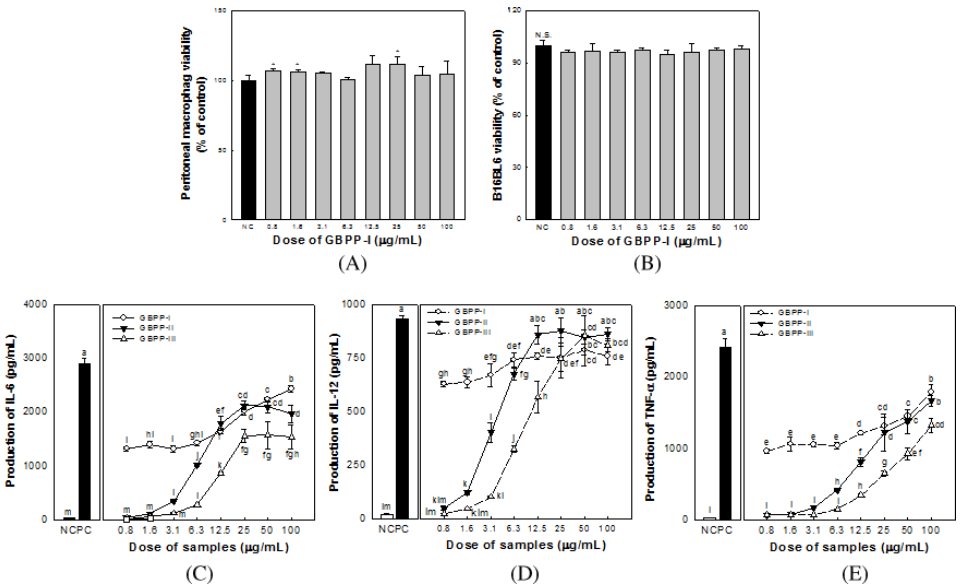
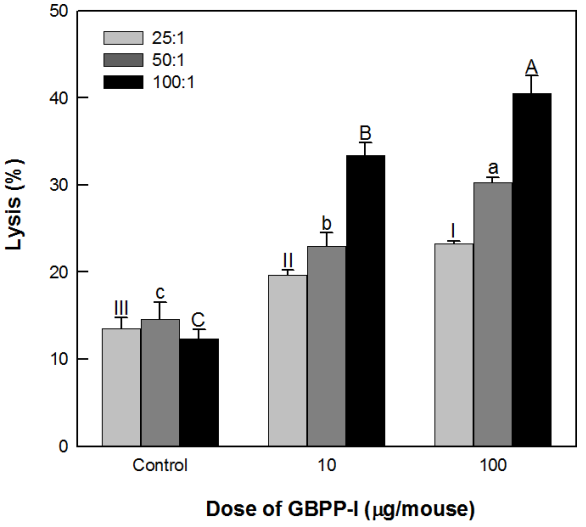


Figure 3. Cytotoxic effect of GBPP-I purified from ginseng berries in peritoneal macrophages (A) and B16BL6 melanoma cells (B) and the effects of GBPP-I, II and III on cytokine production in murine peritoneal macrophages (C–E). Values are expressed as a mean ± SD of three independent experiments performed in triplicate. $^{a-m}$Bars not sharing the same superscript are significantly different from each other ($p < 0.05$).

33-fold compared with a negative control (Figs. 3C and 3D). In addition, the levels of IL-6 and IL-12 were lower than levels induced by 1 μg/mL lipopolysaccharide (LPS), which indicated that inflammation pathways were not involved. IL-6 is also considered to be a major immune and inflammatory mediator (Tanigawa *et al.*, 2000). TNF-α produced a large increase, approximately 55-fold at 100 μg/mL (Fig. 3E). Therefore, GBPP-I was used in subsequent assays.

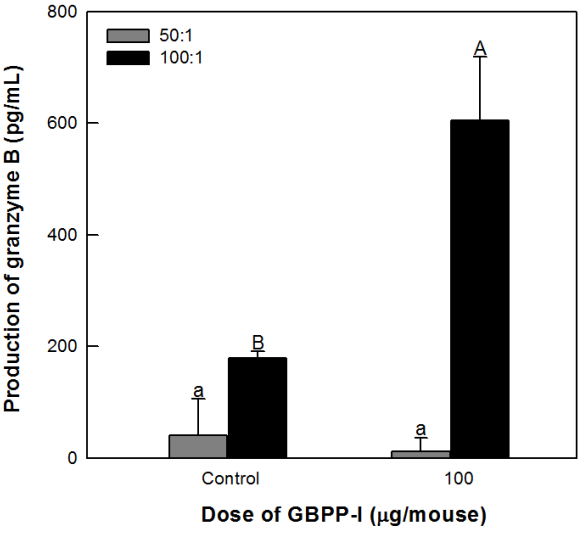
Increased NK Cell Activity after Oral Administration (p.o.) of GBPP-I

YAC-1 cells were used to investigate activation of NK cells after oral administration of GBPP-I. YAC-1 cells are lymphoma cells derived from the Moloney murine leukemia virus lacking expression of major histocompatibility complex (MHC)-1 and are, therefore, highly sensitive to NK cell mediated cell lysis (Kiessling *et al.*, 1975). NK cell-mediated cytotoxicity was determined in YAC-1 cells co-cultured with NK cells obtained from GBPP-I-administered mice (*p.o.*) using an LDH release assay. Splenocytes obtained after oral administration of GBPP-1 to mice at 10 and 100 μg/mouse showed a higher cytolytic activity in YAC-1 cells than in cells obtained from PBS-treated mice (normal control group) in proportion to the E/T ratio (Fig. 4A). Compared with NK cells from control mice, cells from GBPP-1-treated mice showed a significantly higher cytolytic activity than YAC-1 cells (2.71 and 3.29-fold at 10 and 100 μg/mouse concentrations, respectively).



(A)

Figure 4. Effect of oral administration of GBPP-I on cytolytic activity (A) and granzyme B (B) production in NK cells *in vivo*. Values are expressed as a mean ± SD of three independent experiments performed in triplicate. $^{A-C, a-c, I-III}$Bars not sharing the same superscript are significantly different from each other ($p < 0.05$).



(B)

Figure 4. (*Continued*)

GBPP-I also increased granzyme B secretion by NK cells at all E/T ratios. Granzyme B secretion increased after 100 μg/mouse oral administration of GBPP-I. Oral administration of GBPP-I at concentrations of 100 μg/mL increased the level of granzyme B by 3.38-fold at an E/T ratio of 100:1 (Fig. 4A).

Antimetastatic Activity of GBPP-I

To measure the antimetastatic activity of GBPP-I, B16BL6 melanoma cells were used as a representative lung metastatic cancer cell line. Both orally and *i.v.* treated GBPP-I-treated mice showed a dose-dependent decrease in tumor metastasis. GBPP-I delivered by *i.v.* suppressed metastasis by approximately 45% at 100 μg/mouse, compared with control mice (Fig. 5A). Orally administered GBPP-I suppressed metastasis by approximately 39% at 100 μg/mouse compared with control mice (Fig. 5C).

To determine whether the antitumor activity of GBPP-I was due to NK cell stimulation, the rabbit anti-asialo GM1 antibody was used to block the NK cell function (Suttles *et al.*, 1986). An antitumor activity test using B16BL6 melanoma cells was then performed. The number of metastatic colonies in the tumor control group was 55, whereas the number of metastatic colonies in the NK cell blocking group treated with rabbit anti-asialo GM1 antibody was 122, which was a 2.2-fold difference (Fig. 5A). Thus, NK cells play an important role in inhibition of tumor metastasis. The number of metastatic colonies in the 100 μg/mouse GBPP-1 treated group was 29. In addition, the number of metastatic colonies in the GBPP-1-treated group of NK cell-blocked mice was 77, which was reduced by 35% compared with the untreated control group in NK cell-blocked mice.

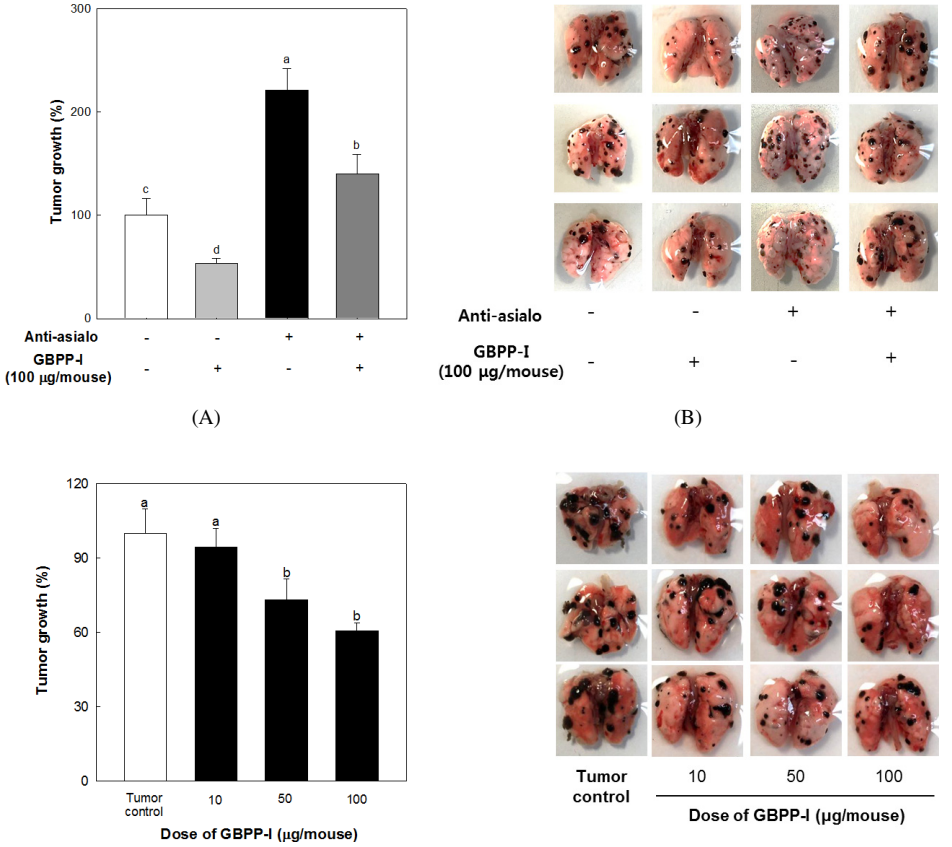


Figure 5. Effect of NK cell activity on GBPP-I-mediated anti-metastasis and the inhibitory effect of GBPP-I on lung cancer metastasis. Values are expressed as a mean ± SD of three independent experiments performed in triplicate. [a–d]Bars not sharing the same superscript are significantly different from each other ($p < 0.05$). Representative photographs of lung tissues (B). BALB/c mice were orally administered with indicated doses of GBPP-I for 15 days before *i.v.* inoculation with B16BL6 melanoma cells. Mice were sacrificed 14 days after tumor inoculation (C). Growth rate (%) of lung tumors, compared with a tumor control (100%). Photographs of excised lungs from each group (D). Values are expressed as a mean ± SD of three independent experiments performed in triplicate. [a–d]Bars not sharing the same superscript are significantly different from each other ($p < 0.05$).

Discussion

As one of the major polysaccharides present in tracheophytes, pectic polysaccharides are the most abundant component of cell walls in dicotyledons, including ginseng. Pectic polysaccharides are soluble and composed of homogalacturonan (HG), rhamnogalacturonan (RG)-I and RG-II domains covalently bound to one another (Paulsen and Barsett, 2005; Voragen *et al.*, 2009). Chemical analysis indicated that GBPP subfractions contain mainly pectic substances, and complex high molecular mass glycosidic macromolecules in the unlignified cell wall are the major components of the middle lamellae in plants

(Voragen *et al.*, 2009). In previous studies, different pharmacological activities have been observed in pectic polysaccharides isolated from plants, and the activities appeared to be dependent on the RG-I and RG-II regions rather than on the HG region (Srivastava and Kulshreshtha, 1989; Paulsen and Barsett, 2005). It has generally been expected that RG-I and RG-II are conjugated with homogalacturonan since RGs have been released from cell walls by endo-polygalacturonase (endo-PGase) digestion (Albersheim *et al.*, 1994). GBPP subfractions, however, were obtained from ginseng berry without endo-PGase digestion. It is possible that the berry contains a potent endo-PGase activity and, therefore, RG-I and RG-II may be generated as a free form by the endogenous enzyme during ripening of the berry.

Among three GBPP subfractions, GBPP-I showed a strong anticomplementary activity herein. The complement system plays an important role in the humoral immune response, which provides protection against bacterial and viral infection, contributes to cancer suppression and mediates phagocytosis of extracellular pathogens via cellular immunity. This immune response is, in itself, an inexpressible but complementary component that affects other immune systems (Shin *et al.*, 1992). A cytotoxicity assay is crucial to investigation of antitumor strategies for eliminating cancer cells with minimal toxicity to normal cells (Kunou and Hatanaka, 1997). GBPP-I does not directly affect tumor cell viability, is not cytotoxic and can induce activation of immune-related cells without toxicity.

One of the major mechanisms of enhancing the immune system via plant polysaccharides is known to include an ability to influence macrophage stimulation and complement activation (Kraus and Franz, 1992). Macrophages belonging to the innate immune system can secrete the inflammatory mediators TNF-α, IL-6 and IL-12 to initiate innate immune recognition and phagocytosis of pathogens. Hence, the level of secreted cytokines reflects the extent of macrophage activation. In addition, modulation of these systems may affect cellular and humoral immune responses (Beutler, 2004). On the other hand, plant polysaccharides are known to stimulate the immune system via modulation of macrophage activity (Schepetkin and Quinn, 2006). As an inflammatory cytokine, IL-6 plays an important role in activating T cells and rejecting tumor cells (Cheng *et al.*, 2008). Many *in vitro* studies have shown that the cytokines IL-2, IL-12 and IL-18 can activate resting NK cells (Shida *et al.*, 2006). Activated NK cells can lyse or inhibit metastasis and growth of a wide variety of tumor cells (Munder *et al.*, 1998). IL-12, a multifunctional cytokine, is critical for eliciting tumor immunity (Munder *et al.*, 1998).

NK cells are innate immune lymphocytes that play an important role in the early host defense against infectious pathogens and tumors (Fehniger *et al.*, 2007). Among the many functions of NK cells, the focus herein was on antitumor activities because mice suffering depletion of NK cells are more susceptible to chemically induced tumors (Smyth *et al.*, 2001). NK cells can destroy tumors directly without prior sensitization *via* specific antigen receptors through several mechanisms, including the release of cytoplasmic granules containing perforin and granzyme B (Cheng *et al.*, 2013). Granzyme B is a cytotoxic protease in NK cells and $CD8^+$ T cells that is released to kill specific targets (Cao *et al.*, 2007). Based on an assay of NK cells herein, GBPP-I increased NK cell activity. NK cells have many functions, however, the focus herein was on antimetastatic activity. In mouse

models that allow investigations of the relationship between NK cells and cancer metastasis, NK cells have been found to play a critical role in host protection against experimental prostate and melanoma and spontaneous breast cancer-induced lung metastasis (Chow *et al.*, 2012; Sathe *et al.*, 2014; Souza-Fonseca-Guimaraes *et al.*, 2015). Results herein showed that NK cell depletion increased tumor metastasis and partially abolished the antimetastatic effect of GBPP-I, indicating that the inhibitory effect of GBPP-I on tumor metastasis was mainly mediated by NK cell activation (Fig. 5A). Consequently, NK and other immune related cells activated by GBPP-I control tumor metastasis and local tumor growth.

It is well known that antitumor activity initiated via administration of biological response modifiers (BRMs) in an experimental metastasis model is mainly activated by macrophage or NK cell activation (Vitale *et al.*, 2014). Most cancer morbidity is known to be caused by primary tumors and metastasis. Therefore, many researchers have studied prevention and treatment of tumors. BRMs can be applied to cancer immunotherapy for inhibiting tumor growth and metastasis and for strengthening the host defense system (Suto *et al.*, 1994; Yoo *et al.*, 1994; Yoon *et al.*, 2004; Zhao *et al.*, 2005). In many cases, BRM activates immune-related cells, such as NK cells, lymphokine-activated killer (LAK) cells, and macrophages, to control growth and metastasis of cancers (Habu *et al.*, 1981; Sakamaki *et al.*, 1992).

In this study, the immunological activity of GBPP-1, a polysaccharide isolated from ginseng berry was evaluated. GBPP-1 increased production of the cytokines IL-6, IL-12 and TNF-α in macrophages and inhibited tumor metastasis via these macrophages and NK cells. Therefore, polysaccharides derived from natural materials, such as GBPP-1, have excellent immunostimulating activities that can prevent cancer metastasis.

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