Research Article

Interferon-γ-Mediated Natural Killer Cell Activation by an Aqueous Panax ginseng Extract

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Panax ginseng extracts are used in traditional herbal medicines, particularly in eastern Asia, but their effect on natural killer (NK) cell activity is not completely understood. This study aimed to examine the effects of P. ginseng extracts on the cytotoxic activity of NK cells. We orally administered P. ginseng extracts or ginsenosides to wild-type (WT) C57BL/6 (B6) and BALB/c mice and to B6 mice deficient in either recombination activating gene 2 (RAG-2) or interferon-γ (IFN-γ). We then tested the cytotoxic activity of NK cells (of spleen and liver mononuclear cells) against NK-sensitive YAC-1 cells. Oral administration of P. ginseng aqueous extract augmented the cytotoxicity of NK cells in WT B6 and BALB/c mice and in RAG-2-deficient B6 mice, but not in IFN-γ-deficient B6 mice. This effect was only observed with the aqueous extract of P. ginseng. Interestingly, the ginsenosides Rb1 and Rg1 did not augment NK cell cytotoxicity. These results demonstrated that the aqueous P. ginseng extract augmented NK cell activation in vivo via an IFN-γ-dependent pathway.

1. Introduction

Panax ginseng (P. ginseng) roots provide a health food and are used to prepare a range of remedies employed in traditional Asian medicine. P. ginseng has been reported to possess several biological activities including antiaging, antidiabetic, anticarcinogenic, analgesic, antipyretic, antistress, antifatigue, and tranquilizing effects [1–3]. Ginsenosides, saponin molecules that are unique to Panax species, are generally considered to be the primary active pharmacological components of P. ginseng [4]. These compounds are also used as markers for the quality control and standardization of commercially available extracts of Panax species. In twenty different saponins, the ginsenosides, Rb1, Rg1, and Rd1, and the notoginsenoside, R1, are considered to be the major bioactive components [5]. In addition, nonsaponin compounds present in P. ginseng are also reported to have hypoglycemic [6] and antitumor activities [7].

There are a number of reports concerning the immunological effects of P. ginseng. However, it is unclear whether P. ginseng activates or suppresses immune responses. Some reports have shown that P. ginseng augments the activity of natural killer (NK) cells [8–13], which play an important role in innate immunity against infection and tumor development [14, 15]. However, the mechanisms involved in this augmentation of NK cell activity have not been identified. The present study examined the effects of P. ginseng on NK cell cytotoxicity using gene-targeted mice. We demonstrated that an aqueous extract of P. ginseng augmented the cytotoxicity of NK cell depending on interferon-γ (IFN-γ).

2. Materials and Methods

2.1. Mice. All mouse experiments were approved by Animal Care and Use Committee in Juntendo University (number 25008 on 2013/2/14 and number 260012 on 2014/2/7).
Wild-type (WT) male C57BL/6 (B6) and BALB/c mice were purchased from Charles River Japan Inc. (Yokohama, Japan) at 6 weeks of age. IFN-\(\gamma\) deficient (IFN-\(\gamma^{-/-}\)) and recombination activating gene 2- (RAG-2-) deficient (RAG-2\(-/-\)) B6 mice were derived as described previously [16, 17]. All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University.

2.2. Reagents. Aqueous (batch number 67EX0326) and ethanol (50% [batch number 67-X-282] or 95% [batch number 67-X-281]) extracts of \textit{P. ginseng} were provided by Nagaoka & Co., Ltd. (Nishinomiya, Hyogo, Japan). Briefly, the aqueous extract was prepared with water at 85°C for 20 h, and the ethanol extracts were prepared by refluxing with 50% ethanol or 95% ethanol for 16 h. Then, extract solutions are concentrated by evaporation in vacuo to give the final extract products. The Rb1 and Rg1 levels in the extracts were estimated by liquid chromatography spectrometry using area normalization methods (Figure 1). Following the instructions of the Japanese Pharmacopoeia (http://jpdb.nih.go.jp/jp16e/jp16e.pdf), we estimated the concentration of Rb1 and Rg1. Rb1 and Rg1 were purchased from Abcam Biochemicals (Cambridge, UK). Rb1 and Rg1 were mixed according to the constituents of several commercially available \textit{P. ginseng} extracts. Extracts and ginsenosides were orally administered to mice as a suspension in distilled water (200 \(\mu\)L).

2.3. Cytotoxicity Assay. Liver and spleen mononuclear cells (MNCs) were prepared as previously described [16–18]. The cytotoxic activity of MNCs was assessed against the NK cell-sensitive YAC-1 cells, using a standard 51 Cr release assay [16–18]. Briefly, Na\(^{51}\)CrO\(_4\)-labeled YAC-1 cells were coincubated with serial dilutions of MNCs on a round-bottom 96-well plate (Corning Inc., Corning, NY) in 5% CO\(_2\) in air at 37°C for 4 h. Spontaneous release was determined by incubating the target YAC-1 cells in medium, and maximal release was measured by placing the target cells in medium containing 1% Triton X-100. The supernatants were collected to measure radioactivity released during incubation, and the percentage of specific lysis was calculated according to the formula Specific lysis = (experimental release – spontaneous release)/(maximal release – spontaneous release) \times 100. As the positive control of cytotoxic assay, we concurrently examined the NK cell activity after the intraperitoneal injection of IL-12 (eBioscience, San Jose, CA) and/or IL-28 (R&D Systems, Minneapolis, MN), which has been described to augment NK cell cytotoxic activity [18, 19], using the same target cells at the same cytotoxic assay condition.

2.4. Flow Cytometric Analysis. After preincubation with an anti-mouse CD16/32 (2.4G2) monoclonal antibody (mAb) to reduce nonspecific binding of mAbs to Fcy receptors, cell surface molecules were stained with FITC-conjugated anti-mouse CD3 mAb (145-2C11) and phycoerythrin-conjugated anti-NK1.1 mAb (PK136) (for B6 mice) or anti-CD49b mAb (DX5) (for BALB/c mice) and analyzed by FACS Caliber (BD Bioscience, San Jose, CA) [18]. All reagents were purchased from eBioscience.

2.5. Statistical Analysis. Data were analyzed by a two-tailed Student’s \(t\)-test. \(P\) values less than 0.05 were considered statistically significant.

3. Results

3.1. Augmentation of NK Cell Cytotoxic Activity following Oral Administration of Aqueous \textit{P. ginseng} Extract. Oral administration of 20, 40, or 100 mg/kg aqueous \textit{P. ginseng} extract to WT B6 mice for 2 days augmented the cytotoxicity of liver and spleen MNCs against YAC-1 cells (Figure 2(a)). Administration of 100 mg/kg aqueous \textit{P. ginseng} extract for 1 day also significantly augmented the cytotoxic activity, although 1-day intake of 20 or 40 mg/kg of this extract did not augment cytotoxicity (Figure 2(a)). Neither the overall MNC numbers nor the NK cell populations in the liver and spleen significantly increased (Figure 2(b)). Oral administration of aqueous \textit{P. ginseng} extract (50 mg/kg) augmented liver and spleen NK cell cytotoxicity in BALB/c and B6 mice, suggesting that there was no strain difference in this response (Figure 2(c)). None of the mice treated with oral aqueous \textit{P. ginseng} extract showed signs of hepatotoxicity (assessed by measurement of serum alanine aminotransferase and aspartate aminotransferase) or systemic toxicity (assessed by observation of body weight, gross appearance, and behavior) (data not shown). These results showed that NK cytotoxicity, but not NK cell number, was increased by oral administration of aqueous \textit{P. ginseng} extract.

3.2. Requirement for IFN-\(\gamma\), but Not for Acquired Immune Cells, for \textit{P. ginseng}-Induced NK Cell Activation In Vivo. To investigate the contribution of acquired immune cells (T cells, NK cells, and B cells) and IFN-\(\gamma\) on the observed \textit{P. ginseng}-mediated augmentation of cytotoxicity in vivo, we orally administered aqueous \textit{P. ginseng} extract (50 mg/kg) to RAG-2\(-/-\) and IFN-\(\gamma^{-/-}\) B6 mice. Liver and spleen MNCs showed augmented cytotoxicity when WT and RAG-2\(-/-\) B6 mice received aqueous \textit{P. ginseng} extract (Figure 3(a)). However, this treatment did not elevate the cytotoxicity of liver and spleen MNCs in IFN-\(\gamma^{-/-}\) B6 mice (Figure 3(a)), indicating a critical role for IFN-\(\gamma\) in this effect. Neither MNC numbers nor the NK cell populations in the liver or spleen increased, even when cytotoxicity was significantly augmented in RAG-2\(-/-\) B6 mice (Figure 3(b)), consistent with our observations in WT mice. These results indicated that oral administration of aqueous \textit{P. ginseng} extract augmented NK cell cytotoxicity in an IFN-\(\gamma\)-dependent manner that was independent of acquired immune cell responses.

3.3. NK Cell Cytotoxicity Was Not Increased by Rb1 or Rg1. Ginsenosides are the major constituents of \textit{P. ginseng}, and Rb1 and Rg1 are generally considered to be the main effector saponins of the \(>20\) reported ginsenosides [5]. We examined whether Rb1 and/or Rg1 activated NK activity in vivo. WT B6 mice were orally administered with mixtures of Rb1 and Rg1.
that were prepared following analysis of the constituents of several commercially available *P. ginseng* extracts (data not shown). Interestingly, cytotoxic activity was not augmented following oral administration of either Rbl or Rgl (Figure 4).

3.4. An Ethanol *P. ginseng* Extract Did Not Augment NK Cell Cytotoxicity. Both alcohol and aqueous extracts of *P. ginseng* are used in complementary and alternative medicines. Our analyses demonstrated that Rbl and Rgl were present at
Figure 2: Continued.
higher levels in alcohol extracts (Table 1). Thus, we examined the effect of ethanol extracts of *P. ginseng* on NK cell cytotoxicity. Oral administration of the 95% ethanol *P. ginseng* extract to WT B6 did not augment liver or spleen NK cell activity (Figure 5(a)). The 50% ethanol extract appeared to increase cytotoxicity, but this effect was not statistically significant (Figure 5(a)). Ethanol extract influenced neither the number of MNCs nor the NK cell populations (Figure 5(b)). These results suggested that some constituent that was present at higher levels in the aqueous *P. ginseng* extract augmented NK cell activity *in vivo*.

4. Discussion

In this study, we explored the activation of NK cell cytotoxicity following oral administration of an aqueous *P. ginseng* extract to WT, RAG-2−/−, and IFN-γ−/− mice. In RAG-2−/− mice, but not in IFN-γ−/− mice, oral consumption of this extract augmented NK cell cytotoxicity to the same extent as
Figure 3: Continued.
that observed in WT mice. Administration of Rbl, Rgl, or an 
edanol extract of *P. ginseng* did not augment NK cell activity. 
None of the treatments studied affected the MNC or NK cell 
numbers, even when NK cytotoxicity was augmented. This is 
the first report to use gene-targeted mice to demonstrate that 
an aqueous *P. ginseng* extract augmented NK cell cytotoxicity 
in an IFN-γ-dependent manner.

Extracts of *P. ginseng* are orally ingested as one of the 
most common complementary medicines for cancers and 
other diseases, particularly in eastern Asia. The present study 
therefore used a mouse oral administration model to examine 
the mechanisms involved in the effects of *P. ginseng* on 
NK cell activity. *P. ginseng* was reported to augment NK cell 
activity in rodents and humans [9–13]. Interestingly, 
consistent with our observations, synergistic increases in the 
level of serum interleukin-12 (IL-12) and IFN-γ accompanied 
by NK cell activation [10] and NK cell activation with no 
substantial effect on T or B cell responses [11] were reported. 
We hypothesize that *P. ginseng* augments NK cell activity via 
similar mechanisms in humans and mice.

Epidemiological studies demonstrated an association 
between ginseng intake and decreased cancer incidence and 
growth [20, 21]. Administration of *P. ginseng* has also been 
reported to inhibit tumor development and/or metastasis, 
as well as NK cell activation, in mice [9, 22]. The present 
study demonstrated that oral administration of an aqueous 
*P. ginseng* extract augmented NK cell cytotoxicity through a 
mechanism involving IFN-γ. NK cell cytotoxicity and IFN-γ 
are generally regarded as critical effectors of immune surveil-
 lance against tumors [15, 23–25]. It was also reported that 
*P. ginseng* induced T helper 1 (Th1) and macrophage cytokines, 
generating lymphokine-activated killer cells in synergy with 
IL-2 [26]. Thus, cytotoxic immune cells would contribute 
to the antitumor effects of *P. ginseng*. In addition, ginseng 
saponins (such as Rg1) have been reported to inhibit the 
production of inflammatory cytokines, TNF-α and IL-6, 
[27–30], which are critical for inflammation-related tumor 
development [31]. Therefore, the antitumor effects of *P. 
ginseng* would be mediated by both the activation of immune 
surveillance and inhibition of inflammation-related tumor 
development.

The results of the present study suggested that some con-
stituent of the aqueous extract, but not of the ethanol extract, 
augmented NK cell cytotoxicity. Some previous reports...
have demonstrated that *P. ginseng* had immunosuppressant effects [30, 32, 33]. It was previously reported that monocyte function, monocyte differentiation into dendritic cells, and toll-like receptor-mediated dendritic cell activation were attenuated by *P. ginseng* [30, 33]. Notably, alcohol extracts of *P. ginseng* that contained high levels of the ginsenosides, Rbl and Rgl, were used in these reports. An acidic polysaccharide from *P. ginseng* was reported to induce regulatory T cells and ameliorate autoimmune disease [34]. It was reported that two different *P. ginseng* extracts had different immunological effects [12]. The effects of various *P. ginseng* extracts probably differ due to their different levels of active compounds.

A variety of receptors, such as toll-like receptors and C-type lectin receptors, have been identified on macrophages, dendritic cells, and innate immune cells [35, 36]. These receptors are thought to mediate sensitivity to microbes, probiotics, and complementary and alternative medicines [37, 38]. The IFN-γ-dependent and acquired immune cell-independent NK cell activation observed following *P. ginseng* administration is fairly similar to that associated with probiotics and β-glucan [17, 39], indicating that toll-like receptors and/or C-type lectin receptors may mediate the effects of *P. ginseng* on NK cell activation. Moreover, ginsenosides are metabolized by colonic bacteria, and these active metabolites mediate their immune and anticancer effects [18]. Taken together, the range of compounds present in *P. ginseng* extracts, the individual differences in the expression profiles of innate immune receptors in human subjects, and the intestinal bacterial flora would all influence the biological effects of *P. ginseng* in humans. To improve the therapeutic benefits of *P. ginseng*, further studies are required to elucidate the complex molecular mechanisms involved in the biological effects of *P. ginseng*.

### Abbreviations

- IFN: Interferon
- MNCs: Mononuclear cells
- NK: Natural killer
- RAG-2: Recombination activating gene 2
- WT: Wild-type
- IL: Interleukin
- SD: Standard deviation
Figure 5: Activation of NK cytotoxicity by oral administration of aqueous, but not ethanol, *P. ginseng* extract. (a) WT B6 mice (*n* = 3 in each group) were administered with aqueous extract (circle), 95% (diamond) or 50% (triangle) ethanol extracts of *P. ginseng* (100 mg/kg), or the same volume (200 μL) of water (square) on days -2 and -1. Liver and spleen MNCs were prepared and cytotoxicity was analyzed using YAC-1 cells at the indicated effector/target ratios. Data are shown as mean ± SD of triplicates samples of all tested mice. Similar results were obtained in three independent experiments. *P* < 0.05 compared with the control at all effector/target ratios. (b) The populations of liver and spleen MNCs were also analyzed by flow cytometry. The MNC number and % of NK cells are indicated below every panel. Data are shown as mean ± SD of three mice in each group. Similar results were obtained in three independent experiments.
Conflict of Interests

Kazuyoshi Takeda received research funding from Nagaoka & Co., Ltd. Ko Okumura declared no conflict of interests.

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