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Potentiation of natural killer (NK) cell activity by methanol extract of cultured cambial meristematic cells of wild ginseng and its mechanism

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ABSTRACT

Aims: As an alternative strategy to obtain large amounts of ginseng extract with high yield of ginsenosides, we have utilized culture of cambial meristematic cells (CMCs) from wild ginseng. The anti-tumor effects of methanol extract of ginseng CMCs (MEGC) and their action mechanisms were investigated.
Main methods: Mice were intraperitoneally administered with MEGC, and we explored NK cell activity, suppression of in vivo growth of tumor cells and relevant molecule expression.
Key findings: MEGC significantly potentiated NK cell activity and suppressed in vivo growth of B16 melanoma cells. However, we observed no increase in NK cell number and unaltered expression of NK cell-activating (NKG2D) and inhibitory (Ly49, CD94/NKG2A) receptors as well as NK cell activation markers (CD25, CD69, CD119, and CD212) in MEGC-treated group compared to the controls. Instead, MEGC significantly enhanced IL-2 responsiveness in the early effector phase and the constitutive expression of granzyme B.
Significance: Our data indicate that culture of CMCs is an attractive alternative method for sustainable production of ginseng extracts and clinical use. In addition, we have unraveled a novel mechanism underlying the potentiation of NK cell activity and antitumor effect of ginseng extract, in which it upregulates the constitutive expression of cytotoxic mediator(s) and IL-2 responsiveness.

1. Introduction

Korean ginseng (*Panax ginseng* C.A. Meyer) <Araliaceae> has been used as a traditional medicine in Eastern Asia for thousands of years [1]. This root, which is humanoid in appearance, has historically been described to benefit all aspects of the human body as a tonic that promotes vitality and enhances physical performance and resistance to stress and aging [2].

A large number of data have shown multiple bioactivities for ginseng including antioxidant [3], antiobesity [4], antidiabetic [5], anti-inflammatory [6,7] and protective effect against physical [8], chemical [9] and biological [10] stress. Moreover, the application of ginseng is now being extended to the control of viral infections such as common colds [11] and acquired immunodeficiency syndrome (AIDS) [12,13]. The active constituents responsible for these effects include a number of saponins commonly known as ginsenosides and non-saponins such as N-containing substances and fat-soluble components have been defined [14].

The effectiveness of ginseng in cancer prevention and control has been one of the most extensively studied properties with respect to its clinical application. Ginseng demonstrates anticarcinogenic [15], cancer chemopreventive [16,17] and antitumor activities [18] in vitro and in vivo. Numerous reports have suggested that the anticancer activity of ginseng is largely contributed by enhanced cellular immunity, which includes natural killer (NK) cells [19,20] and macrophages [19].

It is assumed that the commercial harvesting of wild ginseng plants has begun during 14th century. Due to growing demand and declining harvests of wild roots, the current supply of ginseng mainly depends on field cultivation. However, ginseng cultivation in fields takes a long time, generally 5–7 years, and requires extensive effort regarding quality control. Furthermore, wild ginseng has traditionally been known to be more effective than cultivated ginseng although no definitive conclusion has been reached. To overcome these problems, plant cell culture has emerged as an attractive alternative.

Various tissue culture techniques are being used to produce large amounts of wild ginseng with an increased yield of ginsenosides in a relatively short time [21]. However, these techniques are often not

Abbreviations: CMC, cambial meristematic cells; MEGC, methanol extract of ginseng CMCs.

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commercially viable due to difficulties associated with culturing dedifferentiated plant cells on an industrial scale. To bypass the dedifferentiation step, some of us previously established the technique of isolating and culturing innately undifferentiated cambial meristematic cells (CMCs) [22]. It has been suggested that these cells can provide a cost-effective and environmentally friendly platform for sustainable production of a variety of important plant natural products. In a previous study, it was revealed that CMCs synthesize 23.8- and 24.1-fold more ginsenoside F2 and gypenoside XVII, respectively, than ginseng roots [22]. However, it remains to be determined whether ginseng CMC preparations possess identical effects exhibited by ginseng roots. In the present study, we established CMC culture from wild ginseng and the anti-tumor effects of methanol extract of ginseng CMCs (MEGC) were investigated. Furthermore, we aimed to gain a better understanding of the molecular mechanisms underlying anti-tumor effects focusing on NK cell activity. Herein, we demonstrate that in vivo MEGC treatment potentiates NK cell activity and enhance constitutive expression of cytotoxic mediator(s) in NK cells.

2. Materials and methods

2.1. Reagents and antibodies

The ginsenoside Rb1(079-02191) and Rd(072-03301) were purchased from Wako (Japan) and the ginsenoside Rg1(00007221) and Re(00007211) were purchased from ChromaDex Inc. (Irvine, CA). Gypenoside XVII standard was isolated at the Plant Stem Cell Institute, Unhwa [22].

For culture media, we used complete IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FBS (Moregate, Melbourne, Australia), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-ME (Sigma Chemical, St. Louis, MO). FITC-conjugated anti-mouse CD3, PE-conjugated antibodies against mouse CD119, Ly49A/D, NK1.1 and NKG2D, and PE-Cy7-conjugated anti-mouse NK1.1 antibodies were purchased from eBioscience (San Diego, CA). PE-conjugated antibodies against mouse CD25 and CD212 were purchased from BD Bioscience (San Jose, CA). PE-conjugated anti-mouse CD69 antibody was purchased from BioLegend (San Diego, CA).

2.2. Preparation and analysis of MEGC

2.2.1. Establishment of ginseng CMC suspension culture

CMCs were obtained from the cambium of *P. ginseng* main roots as previously described [22]. Initial suspension cultures were established by inoculating a sample of 2.5 g (FCW) cultured cells derived from CMCs into 125 ml Erlenmeyer flasks containing 25 ml of Murashige & Skoog (MS) medium [23] containing 1 mg/l 2,4-dichlorophenoxyacetic acid(2,4-D), and 30 g/l sucrose. The flasks were agitated at 100 rpm and at 21 °C in the dark. Subculturing was performed at 14 day-intervals.

For proliferation cultures, 20 l air-lift bioreactors were used, and made with Pyrex glass type. The diameter, height and pore size of the sparger were 2 cm, 0.4 cm and 10 μm, respectively. The aeration rate was 0.08–0.18 vvm 2.35 g/l (DCW) for inoculated CMCs. The working volume was 80–85% of the total capacity, which was 16 l of 20 l air-lift bioreactor. Subculturing of CMCs was performed every 13 days and conditioned medium was re-cycled in the ratio of 25% of the working volume. The growth rate was measured in dry cell weight (g/l) after vacuum filtration and drying of the cells was performed in a dry oven at 70 °C for 24 h.

2.2.2. Preparation of MEGC

Freeze dried CMCs (100 g) were extracted twice with 2 l 100% methanol at room temperature for 24 h. The extract was filtered to remove cellular debris and concentrated in a vacuum to yield a dark brown sticky solid (51.18 g). This crude extract was used in subsequent experiments.

For analysis, freeze-dried CMCs were homogenized and 5 g was measured. They were extracted twice with 30 ml methanol for 2 h and then centrifuged to remove cellular debris. Supernatant was collected and adjusted to 100 ml by adding methanol. This analytical sample was filtered with 0.45 μm membrane filter (Fig. 1).

2.2.3. High performance liquid chromatography (HPLC) analysis for ginsenoside determination

Standard ginsenosides Rb1, Rd, Rg1, Re, and gypenoside XVII were aliquoted in 10 mg and solved in 100 ml of methanol. These standard solutions were stored in a cold chamber until analysis.

Agilent 1200 series HPLC system and three different conditions were used for analysis of the ginsenosides Rb1/Rd, Rg1/Re, and gypenoside XVII (Table 1). The concentrations of the ginsenosides Rb1, Rd, Rg1, Re and gypenoside XVII in CMC extract were calculated as the proportion of the area values of each standard solution concentrations.

2.3. Animals and administration of MEGC

Male C57BL/6 mice at the ages of 7–8 weeks were purchased from the Orient Bio (Sungnam, Korea) and acclimatized for one week prior to use. Rodent laboratory chow and tap water were provided ad libitum and the mice were maintained under controlled conditions at the temperature of 24 ± 1 °C, 50 ± 10% humidity and a 12/12 h light/dark cycle. All procedures were done in accordance with the Republic of Korea legislation on the use and care of laboratory animals and the institutional guidelines of Jeonju University and they were approved by the university committee for animal experiments.

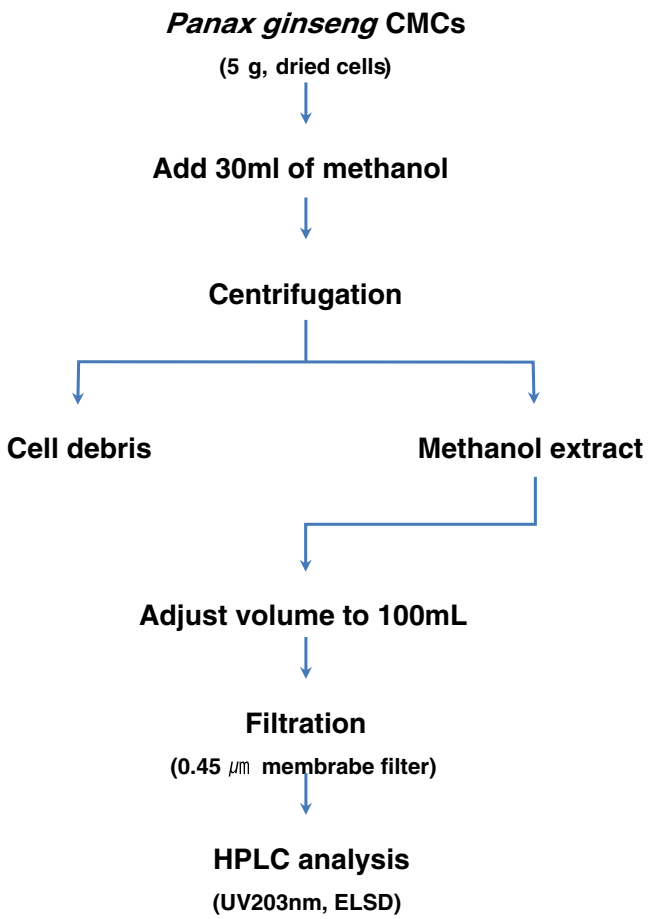

Fig. 1. Preparation of methanol extract of ginseng CMCs (MEGC).

Table 1
Three different conditions for analysis of ginsenosides.

	Ginsenosides Rb1/Rd	Ginsenosides Rg1/Re	Gypenoside XVII
Instrument	Agilent 1200 serise HPLC system		
Column	Capcell pak C_{18} (4.6 mm × 250 mm, 5 μm, Shiseido, Japan)		Watcher 100 ODS-P (4.6 mm × 250 mm, 5 μm, DAISO, Japan)
Mobile phase	A: water (0.05% phosphoric acid) B: 70% acetonitrile (0.05% phosphoric acid)		A: water, B: acetonitrile
Solvent condition	A:B = 57:43, isocratic	A:B = 73:27, isocratic	Water:acetonitrile (for gradient[a])
Detection	UV absorbance at 203 nm (UV detector)		Evaporative light scattering detector (ELSD)
Flow rate	1.0 ml/min		
Column temp.	35 °C	20 °C	20 °C
Injection vol.	20 μl		

Time (min)	Water (%)	Acetonitrile (%)
0	90	10
30	50	50
40	0	100
50	0	100

[a]Mobile phase gradient.

Phosphate-buffered saline (PBS, 200 μl/mouse) containing MEGC or the same volume of methanol as vehicle control was intraperitoneally given daily once a day for 10 days.

2.4. NK cell activity assay

2.4.1. Preparation of spleen cells

Spleens were removed by dissection and spleen cells were obtained by the usual procedure. Briefly, single-cell suspensions were created by teasing with curved needles in chilled medium and erythrocytes were lysed by hypotonic shock. After the cell suspensions were washed by centrifugation at 1800 rev min^{-1} for 5 min at 4 °C, the supernatants were discarded, and the cells were filtered through nylon mesh (70 μm). Adherent cells were removed by incubation at 37 °C for 1 h in complete IMDM medium and non-adherent cells were harvested. The number of viable cells was determined using the trypan blue dye exclusion method and washed cells were resuspended at 2×10^6cells/ml in complete IMDM.

2.4.2. Cytotoxicity assay; 4 h-^{51}Cr release assay

A 4-h chromium release assay was performed as described previously [24] with slight modifications. Briefly, YAC-1 target cells were labeled with 100 μCi of $Na_2^{51}CrO_4$ (Perkin-Elmer Life and Analytical Sciences, Boston, MA) and washed four times with complete medium. The concentration of labeled cells was adjusted to 3×10^5cells/ml, and 5×10^3 cells/well were then added with effector cells (splenocytes) at E/T ratios of 80:1, 40:1 and 20:1 in round-bottom microplates (Nunc, Roskilde, Denmark). After 4 h of incubation, 100 μl of the supernatant was harvested and the released ^{51}Cr was counted in a gamma counter. Cytotoxicity (%) was calculated using the following formula: {(experimental release − spontaneous release) / (maximum release − spontaneous release)} × 100.

2.5. In vivo tumor growth model

B16 melanoma cells (1×10^5 cells in 20 μl) were subcutaneously injected into the footpads of the right hind-limb of animals. Tumor growth was measured with a caliper every other day for 2 weeks, starting on the 8th day of transplantation. Tumor thickness was evaluated by measuring the maximum depth of the tumor within the footpad (mm) and then subtracting the baseline foot thickness of the contralateral, noninjected footpad of the same mouse. The tumor volume (mm^3) was then approximated by multiplying the tumor thickness, maximuml tumor diameter and its perpendicular length as previously described by Fang et al. [25].

2.6. Cell surface marker analysis; flow cytometry

Spleen cells were prepared as previously described and adjusted to a concentration of 1×10^7/ml in PBS containing 1% bovine serum albumin (Sigma, USA). Cell suspensions (100 μl) were incubated with antibodies in the dark on ice for 30 min. For controls, parallel staining was performed using isotype-matched fluorochrome-conjugated Ig (eBioscience). After the final wash, cells were fixed in 1% paraformaldehyde until analysis in a flow cytometer (FACSorter, Becton Dickinson, San Jose, CA) using PCLysys II software.

2.7. Activation of splenocytes with tumor cells for cytokine production and ELISA

Non-adherent splenocytes (1.5×10^6cells/ml) obtained from control and MEGC-treated mice were incubated with 3×10^4 Yac-1 cells exposed to mitomycin C (50 μg/ml, for 30 min) and 12.5 ng/ml of murine recombinant IL-2 (Life Technologies) for 3 days. The IFNγ and IL-10 levels in culture supernatants were determined by ELISA using OptEIA antibody sets (BD Bioscience) according to the manufacturer's instructions.

2.8. Semi-quantitative RT-PCR

Total RNA was extracted from fresh splenocytes using the Easy Blue kits (iNtRON Biotechnology, Sungnam, Korea) according to the manufacturer's instructions. Single strand DNA was synthesized from 1 μg of total RNA using the First-Strand cDNA Synthesis Kit (Promega, Madison, WI) according to the manufacturer's instructions. The complementary DNA was subjected to PCR amplification using rTaq Plus 5× PCR master mix (ELPis, Daejeon, Korea) and 1 mmol/l specific primers. The primers used for the perforin cDNA were 5′-AGC CCC TGC ACA CAT TAC TG-3′/5′-CCG GGG ATT GTT ATT GTT CC-3′ and those used for granzyme B are 5′-GCC CAC AAC ATC AAA GAA CAG-3′/5′-AAC CAG CCA CAT AGC ACA CAT-3′ [26]. These primers generated 349 and 213 bp PCR products, respectively. Primers used for mouse β-actin were 5′-GCT GAG AGG GAA ATC GTG − 3′/5-′GGA GCC AGA GCA GTA ATC-3′ (356 bp product). The PCR cycle conditions for perforin were as follows: 94 °C for 5 min/94 °C for 1 min/59 °C for 1 min/72 °C for 1.5 min for 25 cycles, followed by an extension step of 10 min at 72 °C. The cycle conditions for granzyme B and mouse β-actin were as follows: 94 °C for 5 min/94 °C for 1 min/55 °C for 1 min/72 °C for 1.5 min for 23 cycles, followed by an extension step of 10 min at 72 °C. PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized by staining with ethidium

bromide. Messenger RNA levels from RT-PCR data were compared using relative quantification with Image Quant TL software (GE Healthcare Life Sciences, Sweden) according to the manufacturer's instructions

2.9. Statistical analysis

The values were provided as the means ± SEM and group means were compared using Student's *t*-test, in which $P < 0.05$ was considered significant. Tumor growth was analyzed using 2-way analysis of variance (ANOVA) for repeated-measures followed by Student's t test. All statistical tests were carried out using SPSS software (SPSS 21, SPSS Inc., Chicago, IL).

3. Results

3.1. HPLC analysis of MEGC

Ginsenoside Rb1, Rd, Rg1, and Re standards were detected with retention times (Rt) of 26.25, 72.29, 46.93 and 53.10 min by analytical methods I and II, respectively. MEGC was analyzed by same method and matching peaks of ginsenoside Rb1, Rd, Rg1, and Re were shown. Gypenoside XVII was detected with a retention time (Rt) of 29.5 min by analytical method III using an Evaporative Light Scattering Detector (ELSD) (Table 1). The gypenoside XVII matching peak was shown at 29.53 min. The concentrations (mg/g cell) of ginsenoside and gypenoside XVII were measured by the area of matching peaks. The data demonstrated that Rb1 is the most prominent ginsenoside (4.17 ± 0.12 mg/g cell) in MEGC (Table 2) and unexpectedly, gypenoside XVII was detected (1.30 ± 0.09 mg/g cell) as well. While relatively lower levels of Rg1 and Re were present in MEGC, MEGC also contained some minor compounds such as chlorogenic acid, tryptophan, 3-O-feruloylquinic acid, 3-O-coumaroyl quinic acid, linoleic acid and oleic acid (data not shown).

3.2. MEGC enhanced NK cell activity

It has been well documented that ginseng enhances NK cell activity [19,20]. Here, we evaluated the efficacy of MEGC derived from cultured CMCs of wild ginseng. As shown in Fig. 2A, MEGC treatment enhanced NK cell activity in a dose dependent manner and 100 mg/kg was determined to be the optimal dose. The results from repeated experiments with the optimal dose revealed that the NK cell activity in the MEGC-treated group (10.1 ± 0.54%) was significantly higher than that of the vehicle control group (7.0 ± 0.81%), particularly, at a high E/T ratio ($P < 0.05$, n = 8) (Fig. 2B).

3.3. MEGC suppressed tumor growth in vivo

Suppression of tumor growth by ginseng extract has been demonstrated in numerous reports and this effect is largely due to an enhancement in NK cell activity [19,20]. We thus examined whether MEGC-mediated NK activity enhancement leads to the inhibition of tumor growth. MEGC was administered for 10 days before and after tumor cell transplantation and tumor growth was measured every other day. Tumor growth was visible beginning at 8 days after transplantation and it was highly variable between mice. However, MEGC treatment resulted in a significant suppressive effect on tumor growth when the data were analyzed by 2-way ANOVA ($P < 0.05$) (Fig. 3). On days 20 and 22 of tumor transplantation, the tumor volume in the treated group was significantly reduced compared to the control group (8.0 ± 3.5 vs 45.0 ± 19.4 mm^3 and 18.2 ± 8.1 vs 75.9 ± 26.9 mm^3, respectively) ($P < 0.05$, n = 6).

Table 2
Concentrations of ginsenosides in MEGC.

Ginsenosides	Molecular formula	Rt (min)	Concentration (mg/g cell)
Ginsenosides Rb1	$C_{54}H_{92}O_{23}$	26.24	4.17 ± 0.12
Ginsenosides Rd	$C_{96}H_{164}O_{36}$	72.42	0.697 ± 0.019
Ginsenosides Rg1	$C_{42}H_{72}O_{14}$	47.00	0.314 ± 0.0067
Ginsenosides Re	$C_{48}H_{82}O_{18}$	53.07	0.744 ± 0.012
Gypenoside XVII	$C_{48}H_{82}O_{18}$	29.53	1.30 ± 0.09

3.4. MEGC failed to increase the NK cell numbers

To gain a further insight into the mechanisms underlying NK cell activity enhancement by MEGC, we next examined whether MEGC treatment results in an increase in the NK cell number using flow cytometry. As shown in Fig. 4, we were unable to observe an increase in the $CD3^-NK1.1^+$ NK cell proportion and splenocyte numbers in the MEGC-treated group (data not shown) compared to the control group.

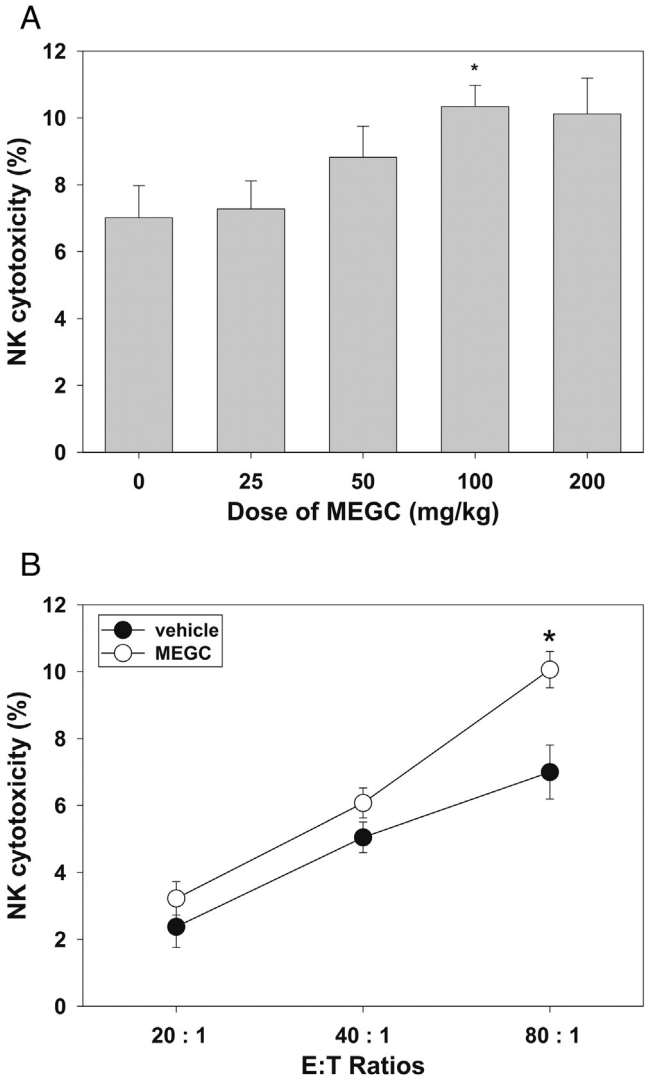

Fig. 2. Potentiation of NK cell activity. PBS containing MEGC or the same volume of methanol as a vehicle control was intraperitoneally given daily once a day for 10 days, and then non-adherent splenocytes were collected. NK cell cytotoxicity was determined using 4 h-^{51}Cr release assay. A dose–response study was performed with 25–200 mg/kg (A). *, $P < 0.05$ vs. vehicle control (n = 5). Accumulated data from repeated experiments using optimal dose (100 mg/kg) of MEGC are shown (B). **, $P < 0.05$ vs. vehicle control (n = 8).

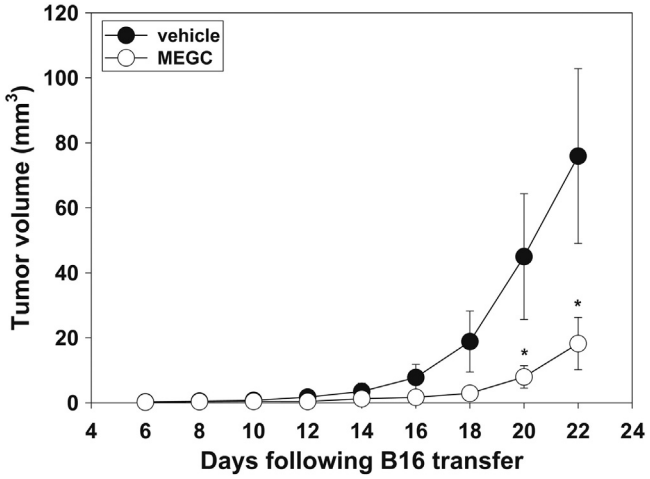

Fig. 3. In vivo suppression of tumor growth by MEGC. MEGC was administered for 10 days before and after tumor cell transplantation and the growth of melanoma in the footpad was measured every other day until day 22. Tumor growth of two groups was significantly different when analyzed by 2-way ANOVA ($P < 0.05$). *, $P < 0.05$ vs. vehicle control (n = 6).

3.5. The expression of NK cell-activating and inhibitory receptors is unaltered by MEGC

NK cell activation and inhibition are controlled by a balance of signals transduced from NK cell-activating and inhibitory receptors [27,28]. Expressional changes in these molecules may enhance NK cell activity. To study this possibility, we examined the levels of lectin-like NKG2D, a NKG2D activation receptor, CD94/NKG2A, an inhibitory receptor that recognize HLA-E and another inhibitory receptor, Ly49A interacting with MHC-I [29]. As shown in Fig. 5, the expression of these molecules in MEGC-treated group was comparable to the control group.

3.6. MEGC failed to alter the expression of the activation molecules on NK cells

Another possible mechanism for the potentiation of NK cell activity is the in vivo activation of NK cells by ginseng extract. If NK cells are preactivated in vivo, they may possess a higher intracellular content of cytotoxic effector molecules which could influence 4 h-cytotoxicity assay results. Thus, we examined the expression levels of activation markers such as CD25 (IL 2 receptor α), CD69 (early T cell activation antigen), CD119 (IFN receptor) and CD212 (IL 12 receptor β1), which are reportedly induced on NK cells following activation. However, there was no difference in the expression of activation markers between vehicle and MEGC-treated groups (Fig. 6).

3.7. MEGC enhances IFNγ production of NK cells

It has long been appreciated that NK cells produce IFN-γ in response to tumor cells [30]. Accordingly, it was hypothesized that MEGC may influence the responsiveness of NK cells to tumor cells. To address this hypothesis, the IFNγ production of NK cells reacting to Yac-1 cells was compared. As shown in Fig. 7A, we were unable to detect IFNγ in the absence of IL-2. While MEGC treatment significantly increased IFNγ content (836.6 ± 149.6 vs. 1329.6 ± 154.6 pg/ml) ($P < 0.05$, n = 7), it had no effect on IL-10 (Fig. 7B), which is produced by splenocytes in the presence of IL-2 after 1 day of culture. However, this effect was not observed after day 2.

3.8. MEGC increases constitutive expression of cytotoxic mediators in NK cells

Our data (Fig. 7) demonstrates that MEGC influences NK cells only in the early effector phase, which suggests that MEGC may not exhibit favorable effects on activation pathway but may rather regulate constitutive function of NK cells. Granzymes and perforin, which can act synergistically are the cytotoxic mediators of NK cells [31] and they constitutively express mRNAs encoding granzyme B and perforin [32]. We thus investigated the expression of these cytotoxic mediators in fresh splenocytes. As summarized in Fig. 8, granzyme B expression in MEGC-treated group (278.8 ± 6.0%) was significantly higher than that in the control group (184.3 ± 30.9%) ($P < 0.05$, n = 4). A similar trend of increase in the expression of perforin was also observed in the treated group although there was no statistical significance due to high variability between the subjects (228.5 ± 33.4% vs. 142.8 ± 26.7%) ($P = 0.09$, n = 4)).

4. Discussion

Wild ginseng has been regarded to be more effective than cultivated ginseng. However, very little data have been accumulated until now due to its limited availability and high cost. Although no significant difference in saponin composition was observed between wild and cultivated ginseng, Tanaka [33] reported that the content of the ginsenoside-Rb1 is

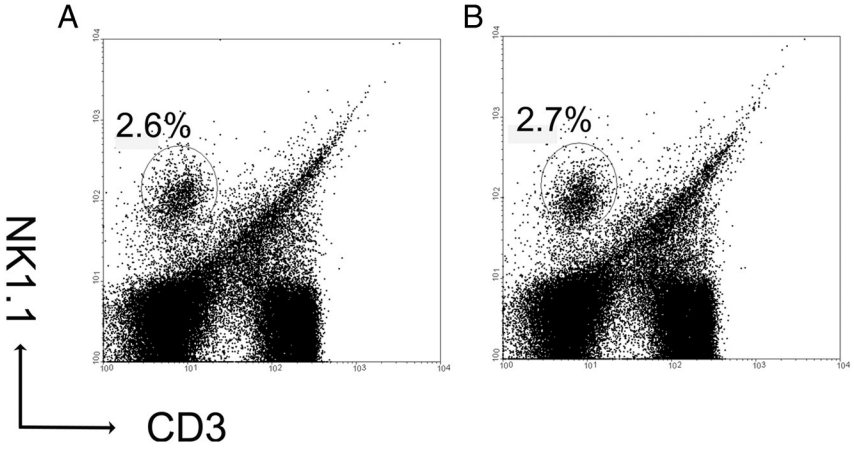

Fig. 4. Comparison of the NK cell proportion in splenocytes between vehicle control (A) and MEGC-treated groups (B). CD3-NK1.1$^+$ NK cells were analyzed by flow cytometry and the data shown are representative of five separate experiments demonstrating similar results.

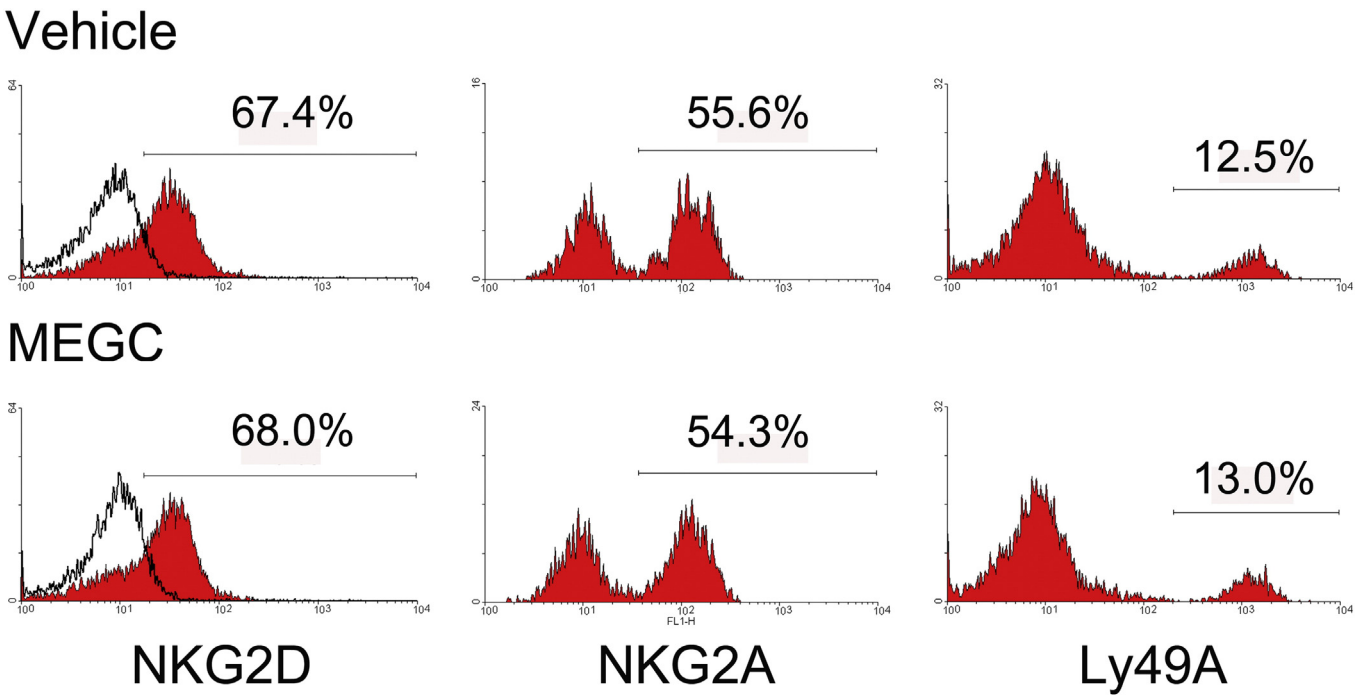

Fig. 5. Comparison of NK cell expression of activation and inhibitory receptors between vehicle control and MEGC-treated groups. Expression was analyzed by flow cytometry and the data shown are representative of four separate experiments demonstrating similar results.

higher in every part of wild ginseng. In addition, the content of malonyl–ginsenoside–Rb1 and ginsenoside–Ro, the saponin of oleanolic acid, in rhizomes was found to be nearly 2-fold higher than that in cultivated ginseng. However, the functional significance of these differences remains to be elucidated.

In the current study, we have observed the characteristics of MEGC distinguishable from cultivated ginseng roots, which are lower content of panaxatriol type compounds (Rg1 and Re) and the existence of gypenoside XVII. Gypenoside XVII is a compound that is not or, if any, only rarely present in cultivated ginseng root and it can be reportedly synthesized by transformation in the fermentation process [34]. However, this compound was detected in MEGC without any further processing or treatment of cultured CMCs. In accordance to this finding, gypenoside XVII was also isolated in suspension cell cultures established from the root of *Panax japonicus* var. *repens* [35], suggesting that its occurrence is not limited to wild ginseng cell culture. The function al significance of gypenoside XVII in ginseng products is an intriguing prospect for future examination.

The primary aim of this study was to evaluate the applicability of cultured CMC which originated from mountain wild ginseng. Numerous studies have shown that ginseng enhances NK cell activity, and thus, we focused on NK cell and its anti-tumor activity. Our data showed that extracts prepared from cultured wild ginseng CMCs is effective for in vivo potentiation of NK cell activity (Fig. 2) and suppression of tumor growth (Fig. 3).

Another significant finding of this study is that we revealed for the first time that ginseng upregulates the expression of cytotoxic mediators such as granzyme B. In order to gain further insight into the mechanisms underlying NK cell activity potentiation by MEGC, we first examined the number of NK cells by flow cytometry and the results demonstrated that MEGC does not increase the number of NK cells (Fig. 4). These results are in contrast with a recent report that ginseng extract increased NK cell numbers [36]. It is unclear whether this discrepancy is due to their experimental design in which long term dietary supplementation and withdrawal for 2 months prior to NK cell counting was used. The difference in the ingredients between two species of the plants used in these two studies may be another explanation for the different results. Though American ginseng (*Panax quinquefolius*) and Korean ginseng (*P. ginseng*) contain similar active ingredients called ginsenosides, the numbers of ginsenoside types contained in Korean ginseng (38 ginsenosides) are substantially more than those in American ginseng (19 ginsenosides). Furthermore, Korean ginseng has been identified to contain more main non-saponin compounds, phenol compounds, acid polysaccharides and polyethylene compounds than American ginseng [37].

NK cell activity is controlled by the balance of signals transduced from NK cell-activating and inhibitory receptors [27,28]. Therefore, changes in the expression of these molecules may enhance NK cell activity. However, data from our flow cytometric analysis showed that the expression of the typical activation receptor (NKG2D) and inhibitory receptors (CD94/NKG2A and Ly49A) on NK cells was unaltered by MEGC treatment (Fig. 5). Several molecules can stimulate NK cells and NK cells that preactivated in vivo may exhibit higher cytotoxic activity in 4 h-^{51}Cr release assay. Whether NK cells are prestimulated in vivo by ingredients in MEGC can be determined by analysis of the expression of activation markers such as CD25, CD69, CD119 and CD212. However, flow cytometric analysis demonstrated no difference in the expression level of those activation markers, between vehicle and MEGC-treated group (Fig. 6). We thus could rule out the possibility that the ingredients in MEGC may activate NK cells in vivo and show higher cytotoxicity ex vivo.

During NK–target cell interaction, signals transduced from the recognition of tumor cells also trigger another event, i.e., secretion of cytokines such as IFN-γ. IFN-γ in culture supernatant was detectable only in the presence of IL-2 and the level of IFN-γ was significantly higher in MEGC-treated group (Fig. 7A). We found no difference in IL-10 secretion, which supports the idea that MEGC is not effective for overall activation of NK cells (Fig. 7B). The mechanism underlying MEGC-mediated upregulation of IL-2 responsiveness in NK cells was currently unknown. NK cells have been revealed to express IL-2 receptors constitutively [38] and MEGC may enhance IL-2 receptor expression on NK cells. However, our data in Fig. 6 indicate that this is not the case and suggest that MEGC affects IL-2 receptor downstream signaling.

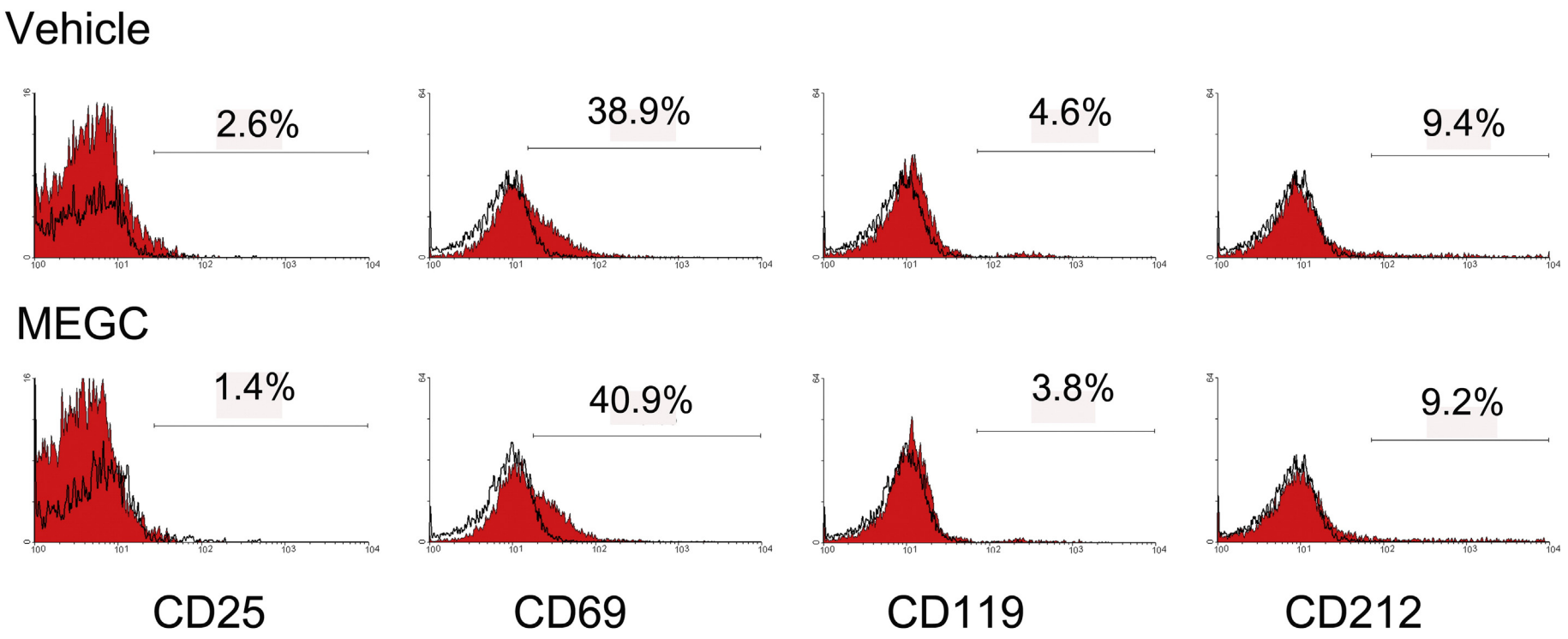

Fig. 6. Comparison of NK cell expression of activation markers between vehicle control and MEGC-treated groups. Expression was analyzed by flow cytometry and the data shown are representative of four separate experiments demonstrating similar results.

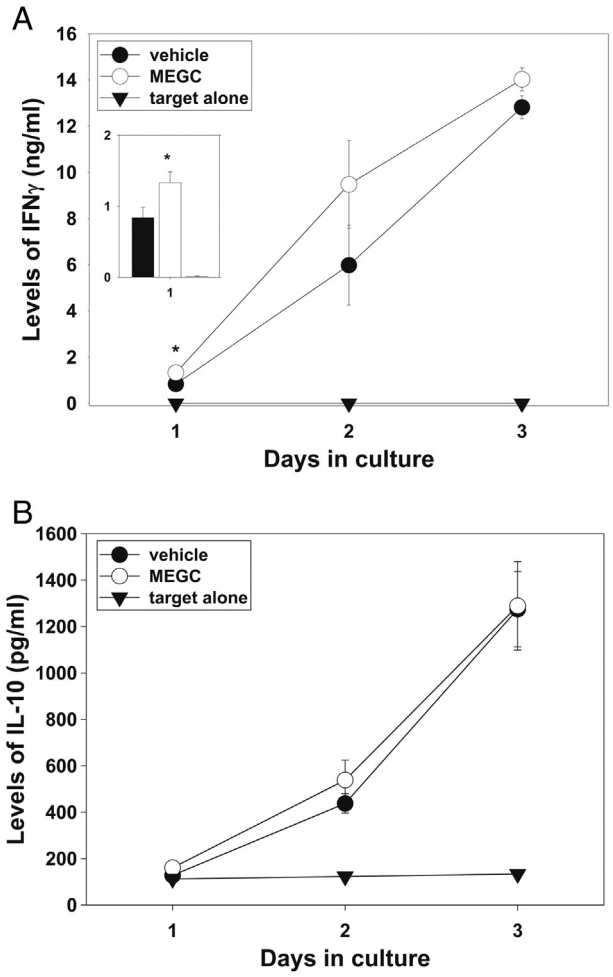

Fig. 7. Effects of MEGC on NK cell cytokine production. Non-adherent splenocytes were incubated with mitomycin C-treated Yac-1 cells and 12.5 ng/ml of IL-2 for 3 days. The levels of IFNγ (A) and IL-10 (B) in culture supernatants were determined by ELISA. *, $P < 0.05$ vs. vehicle control (n = 7).

Notably, the significant difference in IFN-γ levels was observed in only the early effector phase. These data raise the possibility that MEGC upregulates the constitutive function of NK cells. Therefore, we examined the expression of cytotoxic mediators in fresh splenocytes and PCR analysis showed significantly higher expression of granzyme B in MEGC-treated group (Fig. 8). Perforin expression was also similarly elevated by MEGC treatment although the difference was not statistically significant. Further investigation of expressional changes in other cytotoxic mediators would be needed to clarify this. Ginseng has also been suggested to suppress tumor growth by direct effects on tumor cell proliferation such as arrest of the cell cycle or the induction of apoptosis [39]. Ginseng also can prevent cancers through antioxidant or anti-inflammatory activity [40]. Uncovering these mechanisms would be crucial for extending the clinical application of ginseng. Whether MEGC exerts antitumor effects comparable to ginseng root in those mechanisms also remains of interest.

A limitation of the current study is that non-adherent splenocytes instead of purified NK cells were used for IL-2 stimulation and the quantification of mRNA expression. However, previous studies have demonstrated that only NK cells constitutively express IL-2 receptors [38] and the lytic machinery [41,42] and that T cells do not respond to IL-2 in the absence of specific antigenic stimulation. These supportive data allow us to rule out the bias contributed by the cytotoxic T cells included in splenocyte preparation.

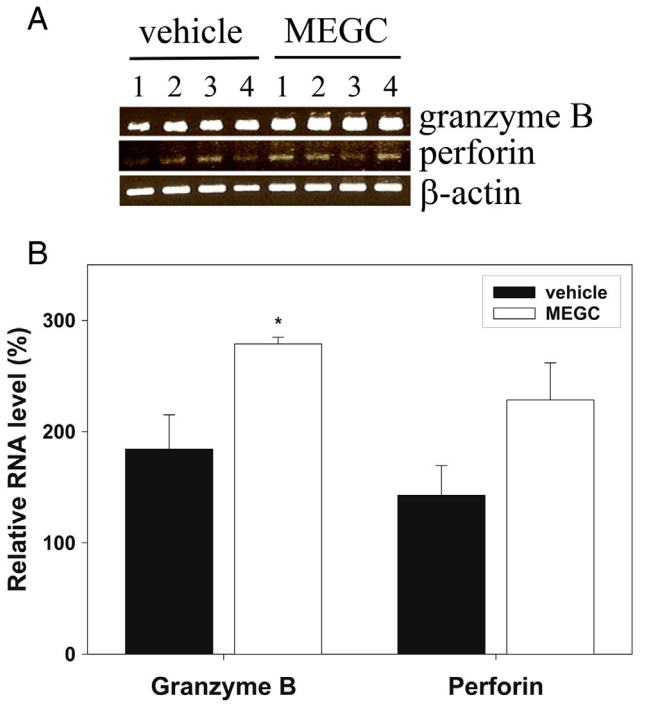

Fig. 8. Effects of MEGC on cytotoxic mediator expression in NK cells. Total RNA was isolated from fresh splenocytes and mRNA levels were analyzed by RT-PCR using the specific primers as described in Materials and methods (A). mRNA levels were compared by relative quantification using Image Quant TL software (B). *, $P < 0.05$ vs. vehicle control (n = 4).

5. Conclusions

Our data indicate that MEGC prepared from cultured wild ginseng CMCs is effective for potentiation of NK cell and anti-tumor activity, suggesting that the CMC strategy is a robust alternative for sustainable production of ginseng extracts. Additionally, the MEGC-mediated enhancement of the IL-2 responsiveness of NK cells in the early effector phase and their elevated constitutive expression of cytotoxic mediator (s) including granzyme B appear to be a mechanism responsible for the efficacy of ginseng in cancer prevention or treatment.

Conflict of interest statement

The authors declare no conflicts of interest.

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