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Induction of secretory and tumoricidal activities in peritoneal macrophages by ginsan

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Abstract

The immunomodulatory effect of ginsan based on the production of cytokines and the activation of macrophage was studied. Murine peritoneal macrophages (PM) on in vitro treatment with ginsan isolated from *Panax ginseng* induced mRNA of cytokines such as tumor necrosis factor (TNF)-α, interleukin-1 (IL-1)β, interleukin-6 (IL-6) and interleukin-12 (IL-12); TNF-α mRNA induction was maximum within 3 h, IL-6 mRNA was gradually induced up to 24 h, and IL-1β and IL-12 mRNA were highly induced at 24 h. IL-1β and IL-6 protein levels also increased within 24 h in a dose-dependent manner and reached a maximum with 100 μg/ml ginsan. IL-12 was induced after 3 days and a high level of induction was detected after 4 days post treatment. Ginsan enhanced the lytic death of L929 cells through TNF-α activation. The mRNA expression of nitric oxide synthase (iNOS) was highly induced after 24 h treatment of ginsan, and then NO production was maximum after 48-h treatment with a low dose of 1 μg/ml. The level of iNOS mRNA induction by ginsan was slightly less than that of macrophages activating agents such as LPS plus IFN-γ. The tumoricidal activity of macrophage cultured with ginsan on Yac-1 cells was enhanced in a dose-dependent manner; growth inhibition increased 1.6-fold with 100 μg/ml ginsan. These results suggest that ginsan exerts as an effective immunomodulator and enhances antitumor activity of macrophages.

Keywords: Polysaccharide; *Panax ginseng*; Activated macrophage; Immunomodulator

1. Introduction

Acidic polysacchride ginsan is an immunomodulatory agent extracted from *Panax ginseng* with a molecular weight of 150,000 Da [1,2]. Previously, we have shown that ginsan was capable in stimulating immune effector cells such as T cells, cytotoxic T lymphocytes (CTL), and natural killer (NK) cells. In addition, ginsan generates MHC-nonrestricted tumoricidal activities from both NK and T cells and augments CTL through the production of multiple cytokines [1–3]. Activated

Abbreviations: PM, peritoneal macrophage; CTL, cytotoxic; T, lymphocyte; NK, natural killer; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PEC, peritoneal exudate cell; RT, reverse transcriptase; CS, cultured supernatant; TdR, thymidine deoxyribose; cpm, counts per minute; LPS, lipopolysaccharide; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate; BRM, biological response modifier.

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macrophages are considered to be one of the important components of the host defense against tumor growth [4]. The activation process includes the generation of cytokines including interleukin-1 (IL-1), tumor necrosis factor (TNF)-α, interleukin-6 (IL-6) and interleukin-12 (IL-12), and they are directly involved in the macrophage-mediated tumor cell killing. The expression of IL-6, having a growth regulatory effect, has been implicated as one of the cytostatic/cytocidal factors in the antitumor action of activated macrophages [5–7]. IL-12 produced by macrophages enhances T-cell responsiveness by producing IFN-γ, and is an immuno-regulatory cytokine that has been shown to generate Th1 and NK response, thereby overcoming tumor-induced immunosuppression.

Nitric oxide is a short-lived radical that is formed by the inducible enzyme nitric oxide synthase (iNOS), and the iNOS is considered to be a central molecule in the regulation of the immune response to tumors. TNF-α, IL-1β and bacterial LPS can induce the expression of iNOS in a wide variety of tissues, organs and in some tumor cell lines. The strong stimulator for the induction of iNOS is LPS, and the induction of iNOS by other stimuli also leads to organ destruction in inflammatory region. iNOS also plays a key role in host defense against infectious agents, including viruses. In the present study, we investigated the effect of ginsan on the activation of macrophages by examining the expression levels and the production of macrophage-induced cytokines and NO. The tumoricidal activity of macrophages cultured with ginsan against L929 and Yac-1 cells was also detected.

2. Materials and methods

2.1. Mice

Inbred strain of 6–7-week-old C3H/HeN female mice were obtained from Charles River Breeding Laboratory (Charles River, Japan), and were maintained in a pathogen-free facility of Korea Cancer Center Hospital.

2.2. Cell culture and reagents

Murine fibroblast cell line, L929, and molony virus induced leukemia Yac-1 were obtained from ATCC (Rockville, MD, USA) and used as tumor target cells for measuring the cytotoxicity. The culture media were RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 2×10^{-2} M HEPES buffer, 2×10^{-3} M glutamine, 1×10^{-3} M pyruvate, 100 U/ml penicillin, 50 μg/ml streptomycin, 5×10^{-5} M of 2-mercaptoethanol and 1% nonessential amino acid (Gibco BRL, Gaithersburg, MD, USA).

2.3. Preparation of ginsan

Polysaccharide 'ginsan' was purified from ethanol insoluble fraction of *P. ginseng* water extract as described previously [1]. Further purification was performed by Sephacryl S-500 gel column (2.6×100 cm) chromatography with 0.1 M sodium phosphate buffer (pH 7.4) and DEAE-sephadex A50 column (3×30 cm) chromatography with stepped gradient of NaCl concentration from 0 to 1 M. Ginsan was composed of $\alpha(1 \rightarrow 6)$ glucopyranoside and $\beta(2 \rightarrow 6)$ frutofuranoside at 3:7 molar ratio, its average molecular weight was 2000 kDa (data are in press). Ginsan was examined based on LAK activity and proliferation of splenocytes, and then the lot exhibiting high activity was used for the following experiments.

2.4. Isolation and activation of peritoneal macrophages (PM)

Thioglycolate-elicited peritoneal exudate cells (PEC) were obtained from C3H/HeN mice by intraperitoneal injection of 1 ml Brewer thioglycolate broth (4.05 g/100 ml) (Difco Labs, Detroit, MI) and lavage of the peritoneal cavity with 5 ml of medium 3–4 days later. PEC were washed twice and resuspended in RPMI-1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Macrophages were isolated from PEC as described by Klimetzek and Remold [8]. Briefly, PEC were seeded at a density of $5–6 \times 10^5$ cells/cm^2 on Teflon-coated petri dishes (100×15 mm) and the macrophages were allowed to adhere for 2–3 h at 37 °C in 5% CO_2 humidified atmosphere. The nonadherent cells were removed and cold PBS (15 ml) containing 1.5% FBS was added, followed by 0.3 ml of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages were harvested. The viability of the detached cells was

assessed by trypan blue exclusion, and the proportion of macrophages was determined by the examination of cytoplasm stained with acridine orange using a fluorescence microscope. Cell preparations were >95.5% viable and contained >90% macrophages [9].

2.5. Semiquantitative RT–PCR

Total RNA was extracted from the untreated or ginsan-stimulated PM by cell lyses in guanidinium isothiocyanate using the RNAzol (Tel-Test, TX, USA). Intact 1 μg total RNA was reversibly transcribed into first strand cDNA, which was then amplified using PCR. The final volume of 20 μl reverse transtriptase (RT) reaction mixture contained: 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl, 2.5 μg/ml oligo $(dT)_{12-18}$, 0.5 mM each of dATP, dGTP, dTTP, and dCTP, and 10 U of AMV-RT (Amersham Pharmacia Biotech, NJ, USA). Ten microliters of cDNA was added to each PCR with the oligonucleotide primers specific for mouse cytokines and β-actin. The oligonucleotide primers used in these experiments are listed in Table 1. The PCR primers were purchased from Bioneer (Seoul, South Korea). The reaction mixture for PCR contained 10 μl cDNA template from RT reaction, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM each of dATP, dGTP, dTTP, and dCTP, 1.0 μM of each primer and 1.25 U Taq DNA polymerase. PCR was performed with a DNA Thermal Cycle (Hybaid, UK) at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min/cycle. The amplified products were electrophoresed in 1% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

Table 1
Oligonucleotides used in RT-PCR analysis

Oligonucleotides		Sequence	Expected size
TNF-α	5′-primer	5′-GCGACGTGGAA CTGGCAGAAG-3′	340
	3′-primer	5′-TCCATGCCGTTG GCCAGGAGG-3′	
iNOS	5′-primer	5′-CCTTGTTCAG CTACGCCTTC-3	499
	3′-primer	5′-CTGAGGGCTC TGTTGAGGTC-3′	
IL-6	5′-primer	5′-TGGAGTCA CAGAAGGAG TGGCTAAG-3′	155
	3′-primer	5′-TCTGACCA CAGTGAGGA ATGTCCAC-3′	
IL-1β	5′-primer	5′-TGAAGGGC TGCTTCCAAA CCTTTGACC-3′	361
	3′-primer	5′-TGTCCATTGA GGTGGAGAG CTTTCAGC-3′	
IL-12p35	5′-primer	5′-ACCTCAGTTTG GCCAGGGTC-3′	500
	3′-primer	5′-GTCACGACGCG GGTGGTGAAG-3′	
β-actin	5′-primer	5′-TGGAATCC TGTGGCATC CATGAAAC-3′	349
	3′-primer	5′-TAAAACGCA GCTCAGTAA CAGTCCG-3′	

2.6. Assay of TNF activity

Cell-free culture supernatants obtained from the treated and untreated macrophages were assayed for the release of TNF activity by determining cytotoxicity using TNF-sensitive L929 fibroblast (ATCC, Rockville, MD) [10]. One hundred microliters of L929 cells (4×10^5 cells/ml) in RPMI 1640 containing 5% FBS were added to 96-well microtiter plates (Nunc, Denmark), and the plates were incubated overnight at 37 °C in 5% CO_2 humidified incubator. After the removal of the medium from each well, 50 μl each of the supplemented EMEM, sample and actinomycin D (2 μg/ml) were added to every well. After 24 h incubation in a humidified CO_2 incubator, the supernatants were discarded and the cells were stained for 10 min with 50 μl of MTT in 20% ethanol. One hundred microliters of 100% methanol was added to each well to elute the stain from the cells. The optical density of each well at 540 nm was determined using Molecular Device microplate reader (Menlo, CA) [11]. Dilutions of recombinant murine TNF-α (Pharmingen, San Diego, USA) were included as a standard. The results are presented as unit per milliliter ± the standard error obtained from three independent experiments.

2.7. Cytokine quantitation

IL-1β, IL-6 and IL-12 were measured in the culture supernatants by the ELISA methods (Quantikine, R&D, Minneapolis, MN, USA), as specified by the manufacturers [12].

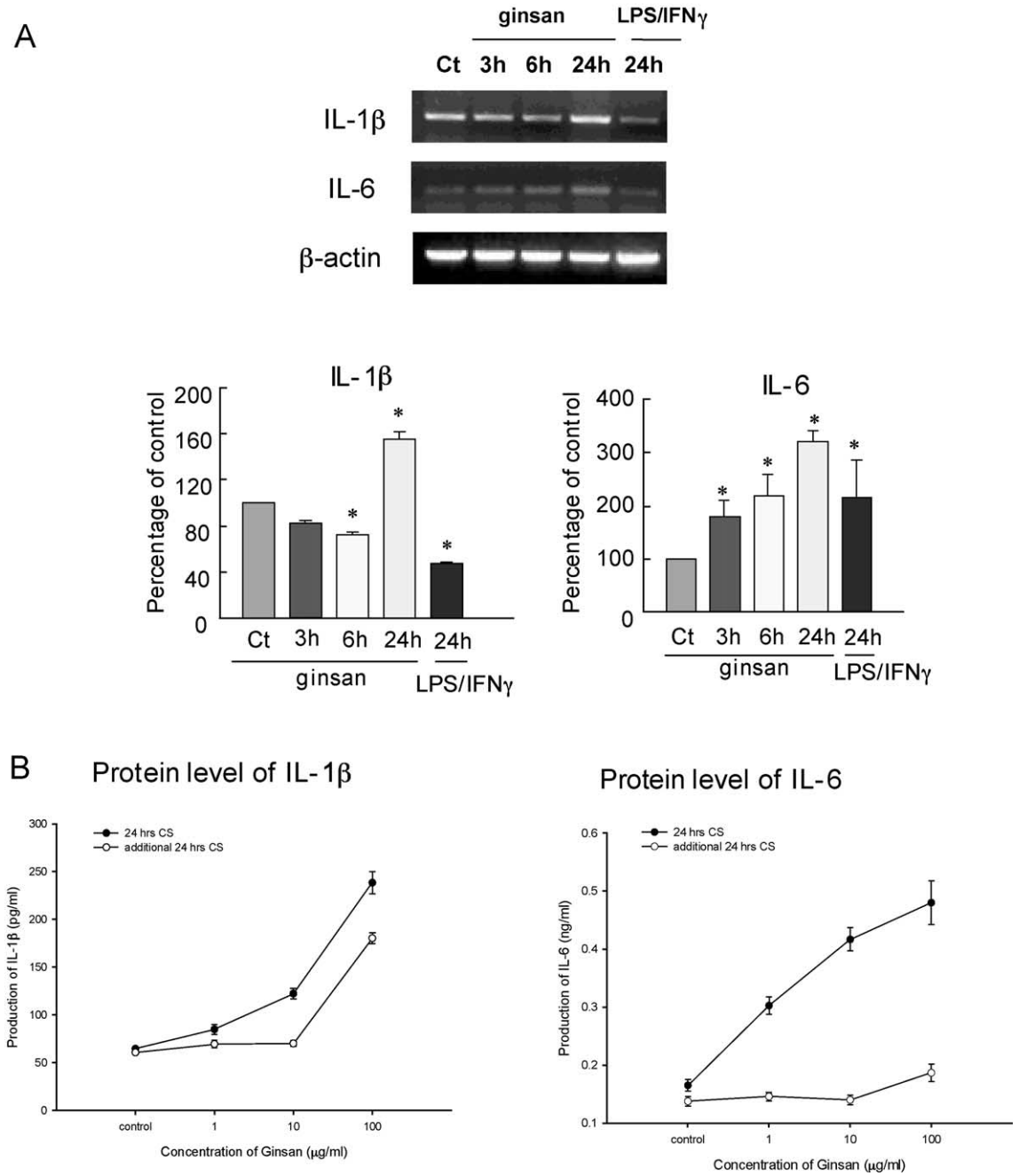

Fig. 1. Proinflammatory cytokine induction by ginsan. (A) Semi-quantitative RT-PCR products from untreated (control) and ginsan (50 μg/ml)-treated PM at various times were analyzed on 1% agarose gel electrophoresis. RT-PCR product from PM treated with LPS (5 μg/ml) plus IFN-γ (50 U/ml) was used as a positive control. The experiment represented one of the three independent trails that gave similar results. The lanes of the gel were quantitated by Image analyzer. The levels of mRNA in PM incubated with ginsan were normalized to control. The data represents the mean ± S.E. of values obtained from three different experiments with duplicate ($*p < 0.05$). (B) PM was treated with ginsan for 24 h. CS was collected directly after a 24 h activation period and fresh medium was added to macrophages for an additional 24 h. Both 24 h CS and additional 24 h CS were assessed for the presence of IL-1β and IL-6 using a specific ELISA. The error bars represent the mean ± S.E. of values obtained from three different experiments.

2.8. Nitric oxide assay

Macrophage cultures were treated with ginsan for 24 h and incubated for additional 24 h in a fresh medium. Both 24 h cultured supernatants (CS) and additional 24 h CS were assessed for nitric oxide production. The accumulation of nitrite in the supernatants was measured by the method of Ding et al. [13]. A total of 100 μl of the supernatant was mixed with an equal volume of Griess reagent for 12 h, and the absorbance at 550 nm was measured using an ELISA photometer. Nitric oxide concentration was calculated from a $NaNO_2$ standard curve. The levels of nitrite are indicative of NO production. Griess reagent was prepared by mixing one part 0.1% naphthylethylene diamine dihydrochloride in distilled water plus one part 1% sulfanilamide in 5% concentrated H_3PO_4.

2.9. Macrophage-mediated antitumor activity

The assay for macrophage cytotoxicity was based on an assay described previously [14]. Cytotoxicity was determined by measuring the radioactivity incorporated into tumor target cells after co-cultivation with macrophages for 24 h. Macrophages (2×10^5 cells/well) were first incubated in either medium alone or in medium supplemented with IFN-γ (50 U/ml) or ginsan (100 μg/ml) for 24 h in 96-well plates. Macrophages were washed three times with RPMI–FBS and co-cultured with Yac-1 tumor cells (2×10^4 cells/well) for additional 24 h at the ratio of 20:1 (PM to Yac-1) in the presence of 2 μCi of [^{3}H]-thymidine. At the end of the incubation, cells were harvested using an automatic multiwell harvester, and the amount of radioactivity incorporated in the target cells was counted in a liquid scintillation counter. Under these conditions, macrophages do not incorporate [^{3}H]-TdR. Cytolytic activity is expressed as the percentage of tumor cytotoxicity where

$$\%\text{cytotoxicity} = 1 - \frac{\text{Target counts/min with macrophages}}{\text{Target counts/min without macrophages}} \times 100.$$

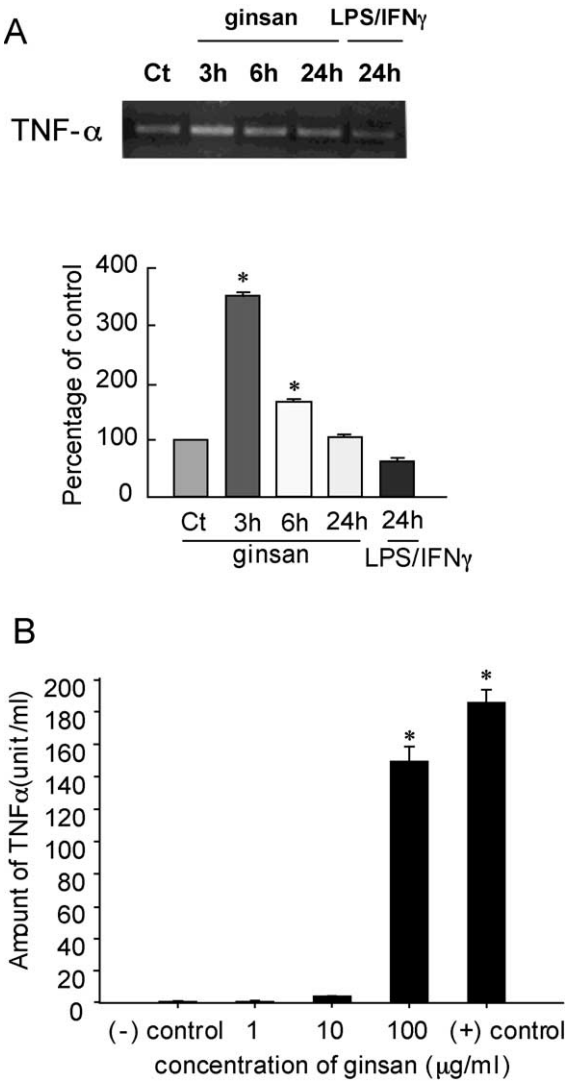

Fig. 2. Induction of TNF-alpha by ginsan. (A) Semi-quantitative RT-PCR products from untreated (control) and ginsan (50 μg/ml)-treated PM at various times were analyzed on 1% agarose gel electrophoresis. RT-PCR product from PM treated with LPS (5 μg/ml) plus IFN-γ (50 U/ml) was used as a positive control. The experiment represented one of the three independent trails that gave similar results. The lanes of the gel were quantitated by Image analyzer. (B) PM was treated with ginsan for 24 h, supernatants were collected and TNF-α in the supernatants was determined by MTT. The optical density of each well at 540 nm was determined using a Molecular Device microplate reader. Recombinant murine TNF-α alone was used as a positive control. The error bars represent the mean ± S.E. of values obtained from three different experiments ($*p < 0.05$).

2.10. Statistical analysis

All experiments were carried out in triplets from two or three preparations, and all values are presented as mean ± S.E. The statistical significance of differences between groups was determined using Student's *t*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Ginsan induced the mRNA expression and the protein level of proinflammatory cytokines

PM was cultured with 50 μg/ml of ginsan and RNA was isolated after 3, 6 and 24 h incubation. PM was also stimulated for 24 h with LPS (5 μg/ml) plus IFN-γ (50

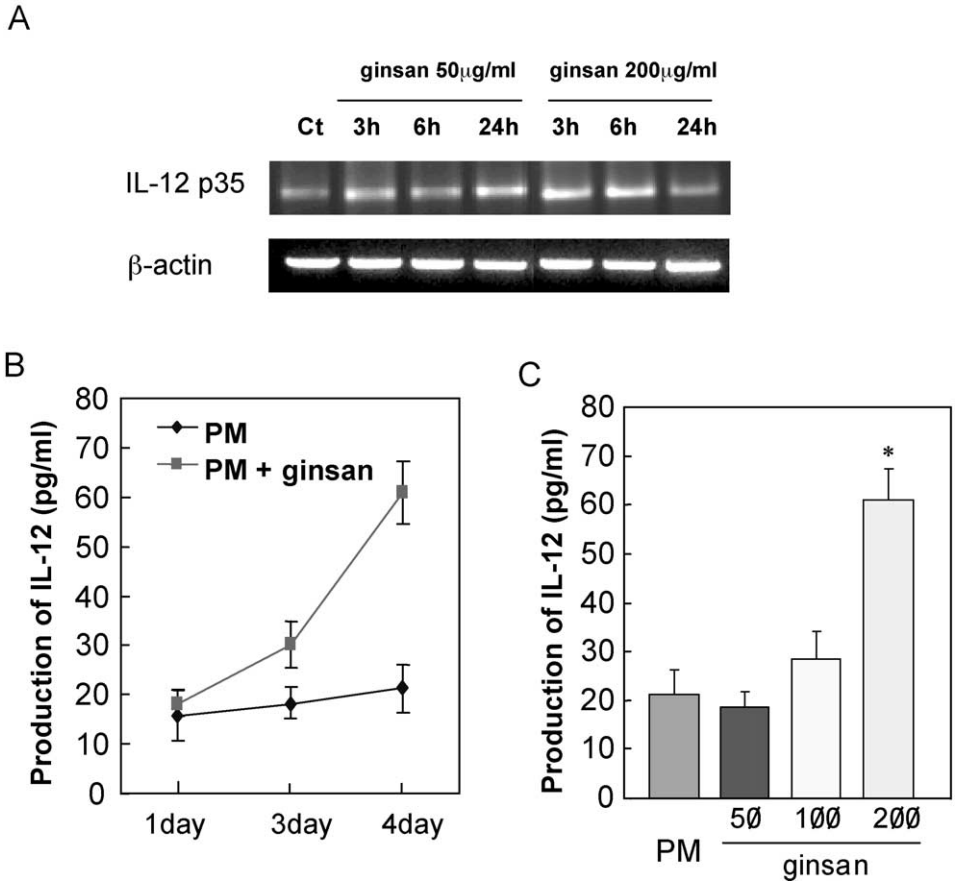

Fig. 3. Dose dependency and kinetics of IL-12 produced from PM cultured with ginsan. (A) RT-PCR analysis of IL-12p35 mRNA level of ginsan-treated macrophages: RT-PCR products from untreated (control) and ginsan (50, 200 mg/ml)-stimulated macrophages at various times were analyzed on 1% agarose gel electrophoresis. (B) IL-12 protein production in ginsan-treated macrophages: PM were isolated from 7-week-old C3H/HeN mice. 2×10^5 cells/well of PM were cultured on 96-well U-bottomed microtiter plate without or with ginsan (200 mg/ml) for 1, 3, and 4 days. The supernatant of culture media was and harvested analyzed for the production of IL-12 by ELISA. (C) PM were cultured on 96-well U-bottomed microtiter plate without or with ginsan (50, 100, 200 mg/ml) for 4 days at 37 °C in 5% CO_2. IL-12 in the supernatant was measured by ELISA. The data represent the mean ± S.E. of values obtained from three different experiments ($*p < 0.05$).

U/ml). The expression of cytokines was determined by RT–PCR and analyzed on agarose gel electrophoresis. As shown in Fig. 1A, not much IL-1β mRNA was induced within 6 h, but up-regulated at 24 h. IL-6 mRNA level was gradually induced up to 24 h (left panel of Fig. 1A). These proinflammatory cytokines mRNA level were not induced after 48 h (data not shown). It is also seen that ginsan is a potent inducer of proinflammatory cytokines, the expression level at 24 h being higher than LPS plus IFN-γ induction. To determine the effect of ginsan on IL-1β and IL-6 secretion in PM, the cells were treated with ginsan for 24 h and further incubated for 24 h in a fresh medium. As seen in Fig. 1B, ginsan stimulated production of IL-1β in PM in a dose-dependent manner with two- and four-fold induction by 10 and 100 μg/ml of ginsan, respectively. IL-6 production was also induced by ginsan in a dose-dependent manner with two- to three-fold induction as compared to the untreated PM. IL-1β was produced with additional 24 h incubation after removal of ginsan; however, IL-6 was not much produced for the additional 24 h incubation.

3.2. Ginsan induced TNF activity in PM

We examined the induction of TNF mRNA and its activity by ginsan in PM. TNF-α mRNA was enhanced at 3 h after ginsan treatment and then slowly reduced to control level at 24 h (Fig. 2A). TNF-α production was determined by assaying a lytic death of L929 cells by the culture supernatant (CS). One hundred microgram per milliliter of ginsan produced as much as 148 U/ml of TNF-α after 24 h incubation (Fig. 2B). On an additional 24 h incubation following the removal of ginsan, TNF-α production was not significantly reduced as compared to the previous day (data not shown).

3.3. Ginsan induced IL-12 in a dose- and time-dependent manner

The effect of ginsan on the expression and production of IL-12 was investigated. IL-12 p35 mRNA level was highly upregulated after 24 h with 50 μg/ml of ginsan and after 3 h with 200 μg/ml of ginsan treatment (Fig. 3A). IL-12 protein production was also enhanced by ginsan after 3 days of treatment and a high level was detected after 4 days (Fig. 3B). IL-12

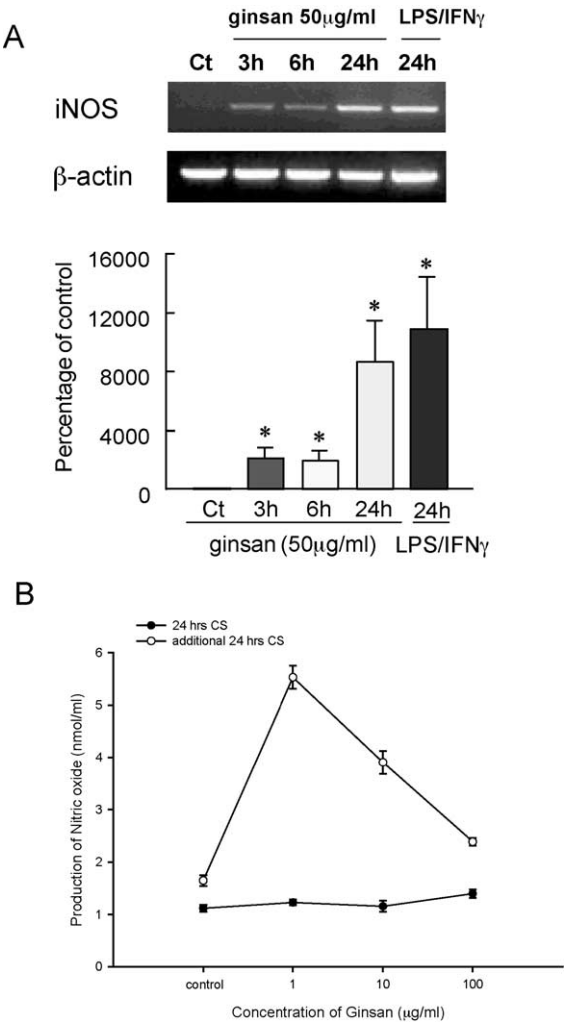

Fig. 4. Ginsan enhanced NO production and iNOS mRNA induction. (A) RT-PCR analysis of iNOS mRNA induction by ginsan: semiquantitative RT-PCR products from untreated (control) and ginsan or LPS (50 mg/ml) plus IFN-g (50 U/ml)-stimulated PM at various times were analyzed on 1% agarose gel electrophoresis. The levels of mRNA in the lanes were quantitated by photoimaging and normalized to control. The data represent the mean ± S.E. of values obtained from three different experiments performed with duplicate ($*p < 0.05$). (B) Effect of ginsan on nitric oxide production by macrophages: PM was treated with ginsan for 24 h. CS was collected directly after a 24 h activation period, and a fresh medium was added to macrophages and incubated for an additional 24 h. Both 24 h CS and additional 24 h CS were assessed for NO using nitric oxide analyzer. The error bars represent the mean ± S.E. of values obtained from three different experiments.

Table 2
Tumoricidal activity by peritoneal macrophages cultured with ginsan

Culture	Dose	[^{3}H]-thymidine incorporation (CPM)	% of inhibition
PM[a]	–	14,174 ± 604	38.1 ± 7.9
PM + IFNγ	50 U/ml	8070 ± 1744	64.8 ± 4.4
PM + GS	1 μg/ml	15,264 ± 1689	33.4 ± 5.6
PM + GS	10 μg/ml	13,533 ± 1689	40.9 ± 6.3
PM + GS	100 μg/ml	8691 ± 925	62.1 ± 3.2
Target[b]		22,915 ± 2871	
Target + GS	1 μg/ml	22,096 ± 2546	3.57 ± 2.3
	10 μg/ml	20,551 ± 190	10.32 ± 1.9
	100 μg/ml	19,386 ± 300	15.40 ± 3.7

PM (2×10^5 cells/well) were isolated from mice and cultured on 96-well U bottomed microtiter plate with ginsan or IFNγ for 24 h. Then, Yac-1 (1×10^4 cells/well) was added and incubated another 24 h in the presence of 2 μCi of ^{3}H-TdR. After culture, Yac-1 was harvested and the inhibition of proliferation of Yac-1 cells was analyzed by ^{3}H-TdR incorporation method.

[a] Peritoneal macrophages.

[b] Yac-1 tumor cells.

was induced by ginsan in a dose- and time-dependent manner at 4 days of post treatment (Fig. 3C).

3.4. Ginsan enhanced iNOS mRNA level and nitric oxide production

As shown in Fig. 4A, 50 μg/ml of ginsan enhanced the level of iNOS mRNA; however, its induction was less than that of LPS/IFN-γ at 24 h. The effect of ginsan on the production of NO in PM was also investigated. Treatment of PM with ginsan resulted in an enhanced production of NO after 48 h. At low dose of ginsan, 1 μg/ml, NO production achieved maximum (Fig. 4B).

3.5. Ginsan enhanced macrophage-mediated cytotoxicity

To assess the cytotoxic activity of ginsan-activated macrophages, untreated or ginsan treated PM were co-cultured with Yac-1 tumor cells. As shown in Table 2, ginsan-primed macrophages enhanced tumoricidal activity in a dose-dependent manner, increasing 1.6-fold with 100 μg/ml of ginsan as compared to untreated macrophages. The degree of antitumor activity of ginsan (100 μg/ml) was similar to that of 50 U/ml of IFN-γ.

4. Discussion

Macrophages are the major source of IL-1, TNF-α, IL-6 and IL-12, and participate as major effector cells in resistance against infectious agents and tumor cells. They are activated to become cytotoxic by a set of cytokine signals. Therefore, in the present study, we examined whether ginsan activated macrophages to induce effector molecules such as cytokines and nitric oxide, and exhibited cytotoxic effect to tumor cells. As demonstrated in Fig. 1A, induction of TNF-α mRNA was seen early in ginsan-activated macrophages. IL-1 and IL-6 mRNA inductions by ginsan were of dose-dependent manner and reached the maximum after 24 h of treatment. It has been known that activated macrophages can kill tumor cells and TNF is responsible for this killing. On the other hand, IL-1 has direct in vitro cytostatic and cytocidal effects. IL-6 is also considered as a major immune and inflammatory mediator. The above three cytokines are related with each other in that they are coordinately released from activated macrophages, and that IL-1 or TNF can induce IL-6 and TNF can induce IL-1. However, IL-6 does not induce IL-1 or TNF, but rather suppresses their production by macrophages. Because the mRNA of TNF-α was rapidly enhanced after 3-h treatment, whereas IL-1β mRNA was significantly induced after 24 h and constitutively expressed in untreated cells, ginsan might be considered to be a strong stimulator of TNF-α rather than IL-1β. IL-6 protein production was not significantly induced after removing ginsan (Fig. 1B), providing a clue that ginsan directly triggered IL-6 protein production, but not through stimulation of IL-1β. IL-12 induces IFN-γ secretion and promotes growth of activated T cell and NK cells, and shows a significant antitumor activity, due to the enhancement of anticancer immunity as well as its anti-angiogenic activity [15,16]. Ginsan enhanced IL-12 mRNA expression in 24 h; however, the protein production was delayed. Further investigation of whether ginsan modifies IL-12 mRNA stability is needed. The cytocidal activity of ginsan might be triggered rather by the production of TNF-α.

TNF-α-induced cytotoxicity may occur by either necrosis or apoptosis, depending on the cell types, and as shown in Fig. 2, ginsan enhanced a lytic death of TNF-α-sensitive L929 cells. Ginsan, 100 μg/ml, was enough to elicit the cytotoxicity and this dose as much

as 148 U/ml of TNF-α activity. L929 cells died by necrosis, possibly due to reactive nitrogen intermediates (RNI) or reactive oxygen intermediates (ROI) produced by mitochondria [17]. The iNOS is induced by a variety of factors including LPS and cytokines (IL-1, IFN-γ, TNF-α). We showed that the mRNA level of iNOS was strongly induced by ginsan at 24 h and its induction was similar to that of LPS plus IFN-γ. The combination of LPS and IFN-γ was considered as a strong iNOS inducer [18]. Ginsan also induced NO production at 48 h by enhancing iNOS expression primarily. However, ginsan-treated cells produced less amounts of NO than LPS plus IFN-γ (data not shown); it might mean that ginsan reduced cytotoxicity towards normal or immune cells around tumor cells.

Macrophage-mediated cytotoxicity was enhanced by ginsan in a dose-dependent manner. Co-incubation of ginsan-activated macrophages with tumor cells either up- or down-regulated the production of different cytolytic molecules depending on tumor cells, thereby modulating susceptibility of these tumor cells to macrophage-mediated tumor cytotoxicity. As shown in this study, ginsan activated macrophages and modulated interaction between tumor and immune cells to enhance the antitumoral activity. Based on the above considerations, we propose ginsan to be a good immunotherapeutic anticancer agent. Either ginsan alone or in combination with chemotherapeutic agents might be applied to cancer patients as an anticancer immunotherapy.

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