Radioprotective Effects of Ginsan Through the Stimulation Hematopoiesis and Antioxidant Enzymes

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Abstract

An immunolodualtor ginsan which was isolated from *Panax ginseng* showed the mitogenic activity, generation of LAK cells, and secretion of several cytokines. In the extended effort to search other immunostimulatory effects, we evaluated protective effects of *in vivo* injected Ginsan against irradiation, by measuring the recovery of CFU-S, and the functioning of bone marrow (BM) and spleen cells. Ginsan was found to significantly increase the number of BM cells, spleen cells, granulocytes macrophage-colony forming cells (GM-CFC), and the number of circulating neutrophils, lymphocytes and platelets in irradiated mice. In addition, it induced endogenous production of cytokines such as IL-1, IL-6, IFN-γ and IL-12, which are required for hematopoietic recovery, and was able to enhance Th1 function while interfering with the Th2 response in irradiated mice. We demonstrated that the pretreatment with Ginsan protected mice from lethal effects of ionizing radiation more effectively than given immediately or after the irradiation. A significant increase of the survival of Ginsan-treated group (100mg/kg) from LD50/30 7.54 Gy of PBS-injection to 10.93 Gy was observed. Moreover, the levels of the antioxidant enzymes such as SODs, catalase and glutathion peroxidase were increased 1.5-2 fold in ginsan treated mice compared to the irradiated mice. These findings indicate that ginsan may be a promising agent to be used in reducing the time needed for reconstituting of hematopoietic cells after irradiation treatment.

1. Introduction

Exposure of mammals to ionizing radiation leads to the development of a complex, dose-dependent series of potentially fatal physiologic and morphologic changes, known as acute radiation syndrome. Radiation-induced destruction of the lymphoid and hematopoietic systems is primary cause of septicemia and death. In the past decades, various substances that
protect the hematopoietic system against adverse effects of cytoreductive treatments have been described. Radioprotectors are either chemical or biological agents, and their administration before or during irradiation diminishes radiation–induced damage. The chemical agents are primarily thiol compounds (1) that are known to act by scavenging free radicals, and the biological agents are immunomodulatory and/or inflammatory substances. Numerous microbial compounds such as bacterial LPS, muramyl dipeptide, Mycobacterium bovis strain bacillus Calmette-Guérin, and glucan have been described to exert radioprotective effects when administered before irradiation (2).

Oriental herbal medicines have been used for the treatment of various diseases for more than 2,000 years, and there is strong evidence that co-administration of herbal medicine with chemotherapy or radiation therapy can reduce the side effects of these treatments and improve general conditions of patients. Accordingly, herbal medicine has recently gained recognition as a biological response modifier (3).

Ginsan is a polysaccharide extracted from the roots of Panax ginseng CA Meyer, and has earlier been reported to stimulate normal lymphoid cells to proliferate and to produce cytokines such as IL-1, IL-2, IFN-γ and GM-CSF (4,5). Since several cytokines have a radioprotective effect, furthermore, GM-CSF, TNF and IL-1 are produced after irradiation in various cells (6) and then these cytokines induced manganese superoxide dismutase (Mn-SOD) mRNA in vitro and in vivo (7), we evaluated that ginsan might exert a radioprotective effect on irradiated mice through the regeneration of hematopoietic stem cells and the induction of antioxidant defense systems.

In the present study, we assessed the in vivo effects of systematic injection of Ginsan on the recovery of hemopoietic damages resulting from sublethal doses of irradiation, the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase in the spleen, and also attempted to elucidate the mechanism of radioprotective activity conferred by Ginsan.

**Materials and Methods**

**Mice.** Six- to 8-weeks old female BALB/c mice were purchased from Charles River Breeding Laboratory (Charles River Japan, Inc. Atsugi Breeding Center, Yokohama, Japan) and were kept for 7 days for acclimatization in our animal quarters. Sterile standard mouse chow (NIH-7 open formula) and water were given *ad libitum*, and they were housed randomly at 60% humidity and 22±2°C on a 12 h light-dark cycle.

**Preparation of Ginsan.** Polysaccharide ‘Ginsan’ was purified from ethanol insoluble fraction of Panax ginseng water extract as described previously (4). Ginsan was dissolved in PBS (pH 7.4), filtered through 0.25μ Millipore membranes and administered intraperitoneally at a concentration of 0.2 ml/mouse 24 h before or after irradiation. Control animals were given 0.2ml PBS at the same time. Hemologic monitoring was conducted 5 and 9 days after irradiation by a cell-DYN 4000.

**Recovery of BM and spleen cells.** Femurs and spleens were removed and placed in PBS solution. Single cell suspensions of BM were prepared by flushing each cavity of the femur
and tibia with 5 ml PBS with sterile syringe and 25-gauge needle. Spleen cells were prepared by mincing and tapping spleen fragments on a stainless 200-mesh in RPMI-1640 medium. Cells were counted by a hemocytometer.

Quantitation of Granulocyte-Macrophage Colony-forming Units (CFU-GM). The BM cells were plated in 35-mm tissue culture dishes (1x10^5/dish) containing Iscove’s Modified Dulbecco's Medium (IMDM, GIBCO, NY) supplemented with 0.3% agar, 20% horse serum and 10% of culture supernatant of NIH3T3 cells engineered to produce GM-CSF by transduction with MFG.GM-CSF retroviral vector. The cultures were maintained in a fully humidified atmosphere in 5% CO_2 at 37°C for 7 days, and the number of colonies consisted of more than 50 cells that had grown in the soft agar was scored.

Assay for CFU-S. CFU-S was determined as described by Till and McCulloch (8). Recipient mice (10 mice/group) were exposed to 9 Gy whole body ^{60}Co irradiation. After 3 hrs, BM cells were pooled from five mice treated with Ginsan and sublethal irradiation 5 days ago, adjusted to 1x10^5 BM in 0.1ml, and injected i.v. into the caudal vein of recipient mice. Seven and 12 days after the transplantation, spleens were removed and fixed in Bouin's solution.

Irradiation. Mice were randomized, placed in ventilated Plexiglas containers and exposed to gamma radiation from the ^{60}Co theraton-780 (Atomic Energy of Canada, Ltd., Canada) at a dose rate of 97.1 cGy/min.

Semiquantitative RT-PCR. The mRNAs of several cytokines and antioxidant enzymes were assayed by PCR amplification of cDNA isolated from spleens of mice. RNA (1mg) was reverse-transcribed in a mixture containing oligo(dT)12-18 primer and superscr ipt reverse transcriptase. The temperatures used for PCR were as follows: denaturation at 95 °C for 1 min; annealing at 55-60 °C for 1 min; and extension at 72 °C for 1 min. The numbers of amplification cycles were determined according to individual primer sets in order to maintain exponential rate of product amplification (30-35cycles). Amplified DNA fragments were subjected to electrophoresis on 1% agarose gel and visualized by ethidium bromide staining.

Tissue preparation and enzyme activity assay. Spleens were removed from mice by section with sterilized scissor. Extracted spleen were homogenized in 9 volume of sucrose/EDTA (0.25 M/1 mM) buffer after washing with saline. Remaining homogenates were centrifuged at 12,000 rpm for 15 min at 4 °C, and the resulting supernatants were analysed for SOD, catalase and GPx activity, using spectrophotometric assays. Protein content was determined according to Bradford method using bovine serum albumin (BSA) as a standard protein. Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) formazan formation at 550 nm by using xanthine oxidase to generate superoxide radical according to the procedure of McCord (9). The reduction of NBT by 50% was defined as 1 unit/mg protein of activity. Catalase activity was assayed by the method of Abei (10), in which catalase activity was determined by the reduction velocity of 10 mM H_2O_2 in 50 mM phosphate buffer, using a spectrophotometer at 240 nm for 3 min. One unit of activity is equal to the moles of H_2O_2 degraded/min per mg protein. Glutathione peroxidase activity was measured by the method of Flohe (11), in which GPx activity coupled to the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) by glutathione reductase. The
oxidation of NADPH was followed spectrophotometrically at 340 nm at 37 °C for 20 min. The reaction mixture was consisted of 1 mM EDTA in 0.1 M phosphate buffer (pH 7.0), 1 mM NaN₃, 1.5 mM NADPH, 10 mM glutathione (reduced form), 0.24 U of glutathione reductase and 1.5 mM H₂O₂. One unit of activity is equal to the mM of NADPH oxidized/min per mg protein.

Statistical analysis. Data were analyzed with one-way ANOVA for significant differences, except for the survival rate of irradiated mice, as mentioned above.

Results and Discussion

Effect of Ginsan on BM and spleen cellularity.

Since the injection time of the radioprotective drug is critical for its activity, we first attempted to establish the optimal time of Ginsan administration for lethally irradiated mice. As shown in Fig. 1, the maximum effect was observed in mice received Ginsan 24 h before the irradiation. A single injection of Ginsan 24 h prior to the irradiation protected 100% of mice from an LD₁₀₀/₂₁ radiation dose (dose of radiation that kills 100% mice in 21 days)(p<0.001). To estimate the dose reduction factor (DRF) of Ginsan, daily mortality was recorded up to 30 days following various doses of radiation exposure, and a significant increase in survival of Ginsan-treated group was observed: from LD₅₀/₅₀ 7.54 Gy of PBS-injection to 10.93 Gy which was calculated using Probit analysis (DRF=1.45). This result indicates that Ginsan has a much superiority to those described for selenium (1.01), glucan (1.22), WR-2721 (1.33) (12), and Zhang et al.‘s fraction (1.17) (13). Based on the above result, ginsan was injected 24 h before irradiation in the following experiments. As seen in Fig. 2A, the number of spleen cells 5 days after the irradiation was only 8% of that of untreated control mice, and it increased to 20% 9 days after the irradiation. However, the administration of Ginsan significantly increased the number of spleen cells by 1.8 and 2.35 fold, respectively, at 5 and 9 days after the irradiation. Although increased from 2.79 ± 0.31 to 6.56 ± 0.97 x 10⁸ cells 9 days after the irradiation, the number of spleen cells was still only 48% of the non-irradiated control mice. The 100mg/kg dose of Ginsan was more effective than the 200mg/kg dose, at 5 and 9 days after irradiation. No effect was observed at the lower and higher doses, these results are similar to other immunomodulator characterized by its bell-shaped dose-response curve (14). The number of BM cells 5 and 9 days after the irradiation was 62% or 80% of the non-irradiated control mice, increasing from 19.73 ± 1.33 to 24.64 ± 2.40 x 10⁶ or 25.19 ± 1.18 to 30.71 ± 2.55 x 10⁶ cells / leg (p<0.05) with Ginsan treatment (Fig. 2B). The data indicated that after sublethal doses of irradiation there was an earlier recovery of spleen and BM cellularity in mice, when injected with Ginsan before the irradiation.

Effect of Ginsan on hematopoietic recovery.

To study the effect of Ginsan on peripheral blood, we also examined the number of blood cells after the irradiation. Five days after the irradiation, the number of white blood cells (WBC), platelet and neutrophils decreased from 3.46 ± 0.24 to 0.48 ± 0.03 x 10⁹/µL, from
585.0 ± 39.0 to 354.5 ± 26.0x10³/µL, and from 0.37 ± 0.04 to 0.04 ± 0.01x10³/µL, respectively. The number of lymphocytes was also significantly decreased. As listed in Table 1, the recovery of WBC in Ginsan-treated mice was 2-fold faster than that of the PBS-treated mice, and especially 4.5-fold faster with neutrophils. After 9 days, the hematological recoveries were similar to those of 5 days after the irradiation. Platelet counts decreased severely in the irradiated mice, however, 1.9-2.5 fold increase was observed in the Ginsan treated group, while Ginsan did not markedly affect the recovery of erythrocyte counts.

**Effect of Ginsan on early recovery of GM-CFC after sublethal irradiation.**

Figure 3 represents the number of GM-CFC 5 and 9 days after the irradiation. Five days after sublethal dose of irradiation, the number of GM-CFU was very low in the PBS-injected mice (10.0 ± 1.1 vs. 59.3 ± 2.3 in nonirradiated mice). However, treatment with Ginsan resulted in a marked recovery of GM-CFC from 10.0 ± 1.1 PBS-injected mice to 47.3 ± 3.9 (p<0.01). The number of GM-CFC in the PBS treated mice decreased more than normal mice (3.3 ± 0.4, compared with 66.8 ± 6.4) at 9 days after the irradiation, whereas Ginsan injected mice showed 5-fold enhancement over the PBS-treated mice (p<0.01).

**Recovery of CFU-S in the bone marrow after 4.5 Gy of irradiation**

To determine the effect of Ginsan injection on the early recovery of CFU-S in BM, mice, given Ginsan 24 h prior to 4.5 Gy irradiation, were sacrificed for CFU-S assays 5 days after the irradiation. As described in Fig. 2 earlier, bone marrow cells of mice treated with Ginsan and radiation were 2.6 fold increased over the non-treated and irradiated mice. As shown in Table 2, the number of CFU-S in the bone marrow of the irradiated control mice was not detected, however, the recovery of CFU-S in the bone marrow of the Ginsan treated group was significantly enhanced.

One might expect that after irradiation a greater number of CFU-S would enhance survival because of faster reconstitution of hemopoiesis. The CFU-S population is very heterogeneous with respect to their physical and biological properties (15, 16), and many have an ‘age structure’ consisted of developmentally early or ‘young’ CFU-S (appearing late, day 12 spleen colonies) with high self-renewal capacity in subsequent divisions and ‘older’ CFU-S (appearing early colonies, day 7) with decreasing self-renewal capacity and increasing differentiation potential (17-19). Therefore, it is suggested that the rapid recovery of GM-CFU after irradiation with Ginsan treatment could be due to both enhanced survival of GM-CFU and differentiation of a greater number of CFU-S.

**Effect of Ginsan on cytokines mRNA expression by gamma irradiation in vivo**

To study relative contribution of various hematopoietic growth factors to the radioprotection conferred by Ginsan, the cytokine expressions of splenocytes were examined at 5 days after the irradiation. As shown in Fig. 4, mRNA expression levels of Th1 type cytokines such as IFN-γ and IL-12 decreased by gamma irradiation, but their decrease was counterbalanced by Ginsan. IL-6 and IL-1β expressions were increased by irradiation, and further increase was observed with Ginsan treatment. Th2 type cytokines, IL-4 and IL-5, were not induced but rather slightly reduced by irradiation with Ginsan treatment (data not shown). On the other
hand, the expression of TNF-α was not altered by both gamma irradiation and Ginsan treatment *in vivo*.

Our preliminary results showed that spleen cells incubated with Ginsan *in vitro* induced a battery of cytokines, including Th1 type (IL-2, -12, IFN-γ), Th2 type (IL-4, -5, -10) and proinflammatory cytokines (IL-1, -6, TNF-α). However in this study, the expression patterns of cytokines by Ginsan treatment *in vitro* and *in vivo* were different. Different sensitivity of immune cells against gamma irradiation was well documented from some studies. Th1 cells are more radiosensitive than Th2 cells (20), NK cells are more resistant than T or B cells, and macrophages are very resistant (21, 22). IL-1 and TNFα have previously been found to protect mice from lethal irradiation, when injected either alone or combination (23). The injection of IL-6 by itself did not improve radiation survival, however, IL-6 has been shown to act synergistically with suboptimal doses of IL-1 to enhance survival from lethal irradiation (24). Moreover, it is well documented that cytokines in combination are more effective to stimulate a broader spectrum of progenitor cells rather than administered separately (25) and endogenously induced cytokines is more effective than their exogenously administered (26). Since Ginsan was able to simultaneously induce a variety of radioprotective cytokines and restore normal cytokine balance broken down by irradiation or cytoreductive drugs, it is suggested that Ginsan might have speeded up the restoration of functional hemopoietic cells by secreting these factors.

**Effect of ginsan on SOD, catalase and GPx enzyme activity**

As shown in Fig. 6, the activity of SOD which scavenges superoxide anion was reduced in irradiation group as compared to the non irradiation group (p<0.05). However, the activity of SOD was significantly increased in the ginsan treated mice group in both sublethal irradiation (p<0.001) and non-irradiation group (p<0.05). Catalase enzyme activity was also decreased after irradiation, however it was increased in the ginsan treated group in both 4.5 Gy irradiation and non irradiation group. On the other hand, GPx activity after irradiation was not significantly changed as compared to unirradiated group. However, the GPx activity was increased by pretreatment of ginsan in irradiated group as compared to irradiated group (p<0.01). In addition, the levels of mRNA and protein expression of these enzymes were exhibited the similar results.

Previous study showed that cytokines such as GM-CSF, TNF and IL-1 were produced after irradiation in various cells (6), and these cytokines induced Mn-SOD mRNA in vitro and vivo (7). Various inflammatory mediators (TNF-, IL-1, IL-6 and LPS) in multiple tissues also have been demonstrated to elicit dramatic elevations of both the mRNA and proteins levels of Mn-SOD (27, 28). Based on our results, the increased expression of Mn-SOD in the administration of ginsan might be due to the increase of TNF and IL-1. In a recent study, catalase is the antioxidant enzyme to be highly expressed in neutrophils (29). As shown in Table 1, the numbers of neutrophils in ginsan treated and irradiated mice were markedly increased by 4.5 fold compared with the irradiated mice. Therefore it could be predicted that the increase of the expression of catalase is partially due to the increased number of neutrophils in ginsan treated irradiation group. On the other hand, it was observed that IFN increases dose dependently the protein levels of GPx and the enzyme activity of Mn-SOD and Cu/Zn-SOD (30). Kandasamy *et al.* reported that IFN and IL-12 increased the expression of GPx (31). In addition, exposure to radiation decreased GPx in hyperthalamus, while
treatment with recombinant human IL-1 increased GPx level. Since ginsan was able to induce various radioprotective cytokines such as IL-1, IL-6, and TNF, it suggest that these cytokines induced by ginsan may be a key role in enhancement of GPx expression.

In conclusion, our experimental results demonstrated that Ginsan conferred a strong radioprotection via two major mechanisms. One was the ability to stimulate CFU-S, the crucial cells for reconstitution of hematopoiesis, toward proliferation and CFU-S self-renewal. The other mechanism of action of Ginsan was to elevate endogenous production of radioprotective cytokines, and then they activated and modulated the antioxidant defense systems.

Further studies on the action mechanisms of Ginsan in radioprotection and the establishment of optimal treatment schedule for the restoration of hematopoiesis in patients treated with radiotherapy are now in progress.

Acknowledgement

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Table 1. Peripheral blood hematology of mice 5 and 9 days after 4.5 Gy irradiation.

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<th>Non-irradiated</th>
<th></th>
<th>Irradiated</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PBS</td>
<td>100mg/kg Ginsan</td>
</tr>
<tr>
<td>5 day WBCs x 10^3/µL</td>
<td>3.46 ± 0.24</td>
<td>0.48 ± 0.03</td>
<td>0.95 ± 0.11*</td>
</tr>
<tr>
<td></td>
<td>0.37 ± 0.04</td>
<td>0.04 ± 0.01</td>
<td>0.18 ± 0.03*</td>
</tr>
<tr>
<td>Lymphocytes x 10^3/µL</td>
<td>2.80 ± 0.26</td>
<td>0.33 ± 0.04</td>
<td>0.68 ± 0.10*</td>
</tr>
<tr>
<td>9 day WBCs x 10^3/µL</td>
<td>8.57 ± 0.12</td>
<td>7.55 ± 0.38</td>
<td>7.94 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>585.0 ± 39.0</td>
<td>354.5 ± 26.0</td>
<td>441.5 ± 38.2</td>
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</table>

Results represent the mean ± S.E of 12 mice per group.
*p<0.001 increase versus PBS. **p<0.01 increase versus PBS.

Table 2. Effect of Ginsan on CFU-S formation.

<table>
<thead>
<tr>
<th></th>
<th>d7 CFU-S/1×10^5 BM cells</th>
<th>d12 CFU-S/1×10^5 BM cells</th>
</tr>
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<tbody>
<tr>
<td>Non-irradiated, control</td>
<td>39.0 ± 0.3</td>
<td>27.2 ± 2.1</td>
</tr>
<tr>
<td>Irradiated, PBS</td>
<td>not detected</td>
<td>not detected</td>
</tr>
<tr>
<td>Irradiated, Ginsan 100mg/kg</td>
<td>17.0 ± 0.3</td>
<td>15.6 ± 0.5</td>
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Balb/c mice (5 mice/group) were i.p injected with Ginsan 24 h before 4.5 Gy gamma irradiation. Five days later, 1×10^5 bone marrow cells of each group were injected i.v. to lethally irradiated syngeneic mice (10 mice/group). The number of colonies in spleen was macroscopically counted 7 and 12 days later, following fixation with Bouin’s solution. All results are mean ± S.E.M.
Fig. 1. Survival enhancing effect of Ginsan in mice that have received lethal irradiation. Balb/c mice received 100mg/kg of Ginsan *i.p.* at various times before or after 8 Gy of irradiation. The percent of survival was calculated from two experiments each with 10-20 mice. * Ginsan was given within 15 min after irradiation.
Fig. 2. Effect of Ginsan on the spleen (A) and bone marrow (B) cellularity 5 and 9 days after sublethal irradiation
Mice were injected with Ginsan or PBS 24 h prior to 4.5 Gy irradiation. Five and nine days after irradiation, femurs and spleens were removed and prepared single cell suspension. Mean ± S.E. (bars) of 3 experiments of 5 mice/group/experiment was represented.

Fig. 3. Effect of Ginsan on the numbers of GM-CFU 5 and 9 days after sublethal irradiation
Ginsan or PBS was injected 24 h before 4.5 Gy irradiation. On day 5 and 9 after irradiation, BM cells were seeded in semi-solid agar cultures containing 10% GM-CSF supernatants as a source of CSF. Data represent Mean ± S.E. (bars) from 3 experiments of 5 mice/group/experiment.
**Fig. 4. Effects of Ginsan on mRNA expression of cytokines in vivo**

Ginsan or PBS was *i.p.* injected to mice (5 mice/group) 24 h before 4.5 Gy irradiation (1: control, 2: Ginsan 100 mg/kg, 3: Ginsan 200 mg/kg, 4: irradiated group (IR), 5: treated with ginsan 100 mg/kg and irradiated, 6: treated with ginsan 200 mg/kg and irradiated). After 5 days, the mice were sacrificed and splenocytes were isolated from each group. Total RNA was purified from the splenocytes of each group and performed RT-PCR and analyzed on 1% agarose gel electrophoresis. The data represents the mean ± SEM of values obtained from two different experiments performed in triplicates.
Fig. 5. Effect of Ginsan on the activity of antioxidant enzyme in the spleen after irradiation.

Five days after 4.5Gy irradiation, the effect of Ginsan on the activities of (A) superoxide dimutase, (B) catalase and (C) glutathione peroxidase were examined in the spleen. Mean ± SEM. (bars) of 3 experiments of 5 mice/group/experiment was represented.

**P<0.01 and ***P<0.001 versus irradiated group
Reference