Enhancement of Repopulation and Hematopoiesis of Bone Marrow Cells in Irradiated Mice by Oral Administration of PG101, a Water-Soluble Extract from *Lentinus lepideus*

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PG101 is a water-soluble extract from *Lentinus lepideus*. It is a potential biological response modifier that activates selective cytokines *in vitro*, mainly by controlling cellular transcription factor NF-κB. Effects of PG101 were tested on bone marrow cells in irradiated mice. Mice were irradiated with a dose of 6 Gy and were given PG101 by gavages daily for 24 days. In PG101-treated mice, the number of colony-forming cells, including colony-forming units (CFU)-granulocytes/macrophages (GM) and erythroid burst-forming units (BFU-E), were increased to almost the levels seen in nonirradiated control as early as 8 days after irradiation. Two-color flow cytometric analysis using antibodies to ER-MP12 and ER-MP20 suggested that in the bone marrow cell population, PG101 increased the number of granulocytes (ER-MP12$^-$20^{med}) and myeloid progenitors (ER-MP12$^+$20$^+$). Analysis of surface c-Kit and Gr-1 proteins in bone marrow cells indicated that PG101 might induce differentiation of progenitor cells to granulocytes and/or proliferation of the committed cells. Lastly, oral administration of PG101 highly increased serum levels of GM-CSF, IL-6, and IL-1β. Interestingly, the level of TNF-α was elevated by irradiation in control mice, but was maintained at the background level in PG101-treated mice, suggesting that PG101 might effectively suppress TNF-α-related pathologic conditions. Our results strongly suggest the great potential of PG101 as an immune enhancer during radiotherapy and/or chemotherapy. Exp Biol Med 228:759–766, 2003

Key words: *Lentinus lepideus*; PG101; hematopoiesis; bone marrow; myeloid progenitor cells; granulocytes; cytokines

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Radiotherapy and/or chemotherapy often results in hematopoietic and immune dysplasia as hematopoietic stem cells are damaged during the procedure, and subsequently, committed hematopoietic and immune cells are depleted. Consequently, patients often experience anemia, lymphocytopenia, thrombocytopenia, and/or granulocytopenia, leading to serious and lethal infections and increasing the mortality and morbidity of these patients. Irradiation affects almost all subpopulations undergoing cell division, including early blasts, present in the bone marrow. Therefore, how rapidly patients recover from radiotherapy and/or chemotherapy greatly depends on the percentage of resting stem cells remaining after such treatment.

As a means to protect stem cells or help damaged stem cells to recover, the use of biological response modifiers (BRMs) has received attention. Various compounds, especially carbohydrates isolated from mushrooms, yeasts, and plants, were reported to affect bone marrow and peripheral blood cells, and to induce hematopoiesis (1–3). For example, a single peritoneal injection of Scleroglucan, derived from *Sclerotium glucanicum*, enhanced the bone marrow cellularity (4), and OL-2 from *Omphalia lapidescens* increased the number of lymphocytes and various immune cells in both the peritoneum and the spleen (5). Intravenous injections of glucan-F, a soluble glucan, increased the overall number of granulocyte/macrophage (GM)-colony forming units (CFU) as well as the erythroid CFU in irradiated mice (6–8). The underlying mechanism of these observations is not yet clear. However, it was reported that carbohydrates with hematopoietic activity (9), such as CARN750 [a soluble β-(1,4)-linked acetylated mannan], isolated from *Aloe barbadenis*, could activate macrophages and monocytes, inducing the production of IL-1, IL-6, interferon, and GM-CSF *in vitro* (2).

We have screened a variety of mushroom extracts for the possible presence of immune-modulating activities.

During this study, we previously found that extracts from *Lentinus lepideus* contain strong anti-cancer activity and that a water-soluble glycan from *L. lepideus* induces B cell proliferation in the mouse system (10). We also have found that a water-soluble extract, PG101, from *L. lepideus* can activate cellular transcription factor NF-κB and control the expression of various cytokines, including TNF-α, IL-1β, IL-10, IL-12, IL-18, and GM-colony-stimulating factor (CSF) in human peripheral blood mononuclear cells (PBMCs) (11). Some of the cytokines, such as IL-1β and GM-CSF, have been reported for their therapeutic effects on hematopoiesis and immune cell by stimulating bone marrow regeneration (12). Therefore, we tested whether this mushroom extract could be used overcome the immune-suppressive situation created by radiation-induced bone marrow damage. It was found that in irradiated mice, the oral administration of PG101 increased the number of CFCs, CFU-GM, and erythroid burst-forming units (BFU-E) almost to the normal levels. Two-color flow cytometric analysis using antibodies to ER-MP12 and ER-MP20 showed that the subpopulation of early myeloid progenitor cells might be the major target of PG101. PG101 also appeared to induce the proliferation of the cells committed to granulocytes because the number of Gr-1-positive and c-Kit-negative cells was highly increased. Increased levels of radioprotective cytokines, including IL-1β, IL-6, and GM-CSF, which are well known to stimulate stem cell recovery and hematopoietic regeneration after bone marrow damage (13–15), were also observed in the serum of irradiated mice treated with PG101. Our data suggests the potential of PG101 as an effective BRM and its possible use as an immune enhancer in immunocompromised and immunosuppressed individuals.

Materials and Methods

Preparation of PG101. PG101 was prepared as described previously (16), and the resulting brown powder was weighed and dissolved in phosphate-buffered saline (PBS) for oral administration for mice. Endotoxin in prepared PG101 was assayed under endotoxin-free experimental conditions using a Limulus Amebocytes Lysate (LAL) Pyrogen kit (BioWhittaker, Walkersville, MD). The quantity of endotoxin in PG101 was less than 0.015 EU/mg.

Animals and Design of the Experiment. Female BALB/c mice, 8 to 10 weeks of age and weighing 18 to 20 g, were purchased from the Seoul National University Laboratory Animal Center. Mice were housed either in conventional cages or, after irradiation, in positive pressure lamina air flow microisolators (Lab Products, Maywood, NJ) on cob chip contact bedding. The animals were housed under specific pathogen-free conditions according to the institutional guidelines. Research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council (http://www.nsa.edu/nrc). For irradiation, mice were placed in ventilated containers and were exposed bilaterally to gamma irradiation from a cobalt-60 source teletherapy unit (Model V9, Picker, Cleveland, Ohio). Exposure time was adjusted so that each animal received a 6 Gy midline tissue-absorbed dose. Mice were first divided into four groups. In the first group, mice were irradiated with 6 Gy, and then 10 mg of PG101 per animal was orally administered every day for 24 days. In the case of the second group, irradiated mice were injected subcutaneously with 3 μg of G-CSF on a daily basis. G-CSF is well known to induce differentiation of bone marrow cells to the granulocyte lineage. The third group was the control in which mice were treated with PBS. The fourth group was nonirradiated and was administrated PBS. Each group consisted of nine mice. Three mice from each group were sacrificed by cervical dislocation on Days 8, 16, and 24 after irradiation for hematological and bone marrow evaluation. The parameters analyzed included the number of the CFU in the bone marrow cells, the number of specific subpopulations as determined by FACS, and serum levels of cytokines. The concentration of PG101 used for the experiment was chosen based on the preliminary experiment showing that the effect of PG101 became significant between 1 and 10 mg per mouse (0.05-0.5 g/kg), which did not show any toxic effects on mice (data not shown).

Antibodies and Conjugates. Biotinylated c-Kit (anti-CD117), Gr-1 (anti-Ly-6G), ER-MP12 (anti-CD31), and fluorescein isothiocyanate (FITC)-coupled ER-MP20 (anti-Ly-6C) antibodies were purchased from BD PharMingen (San Diego, CA). As secondary conjugated antibodies, phycoerythrin (PE)-conjugated streptavidin (SAv-PE; Sigma Chemical Co., St. Louis, MO) was used.

Colony-Forming Assay. Bone marrow cells from intact mice were drawn by flushing femoral bones with an IMDM medium and the femoral bone marrow cells were placed in triplicate at a concentration of 1×10^5 cells/ml in semisolid methylcellulose supplemented with 1% methylcellulose in IMDM, 15% fetal bovine serum (FBS), 10^4 *M* β-mercaptoethanol, 10 ng/ml IL-3, 50 ng/ml SCF, and 3 units/ml erythropoietin (Stem Cell Technologies, Vancouver, British Columbia, Canada) as a source of colony-stimulating activity. Plates were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Colonies were counted under the microscope 10 days after culture. The number of colonies consisting of more than 50 cells was counted, and the average number of colonies and standard errors were calculated from triplicate. The different colonies were identified and counted based on their morphological characteristics as referenced by Murine Hematopoietic Cell Colony Photographs from Stem Cell Technologies. BFU-E is light or pinkish brown color, small in size, dense, and irregular in shape. Colorless colonies with a dark, dense core or those consisting of granular or foamy cells were counted as CFU-GM.

Flow Cytometry Analysis. The femoral bone marrow cells were washed with PBS containing 2% FBS and 0.1% sodium azide (FACS buffer) and were preincubated in the same buffer with 25% normal rat serum for 30 min at

4°C. Cells were reacted with 100-μl aliquots of a mixture containing saturating amounts of biotinylated anti-mouse ER-MP12 for 30 min at 4°C in a dark space. Cells were then washed twice with FACS buffer and were reacted with SAv-PE or FITC-conjugated anti-mouse ER-MP20 for 30 min at 4°C. Cells were washed again and suspended in 500 μl of FACS buffer. For the detection of c-Kit and Gr-1 in bone marrow cells, PE-conjugated anti-mouse c-Kit antibody or PE-conjugated anti-mouse Gr-1 antibody, respectively, was used under identical experimental conditions (30 min, 4°C). Flow cytometry was performed on a FACSort (Becton Dickinson, San Jose, CA) with CellQuest (Becton Dickinson) data acquisition and analysis software. In all cases, nonspecific staining was controlled by isotype-matched antibodies.

Measurement of Cytokine Levels. ELISA kits for TNF-α, IL-1β, IL-6, and GM-CSF were purchased from Endogen (Woburn, MA), and levels of the cytokines were measured according to the manufacturer's instruction.

Statistical Analysis. Data are expressed as means ± SEM. Statistical significance was determined using a Student's *t* test.

Results

Effect of PG101 on Hematopoietic Stem Cells in Irradiated Mice. Because rapidly dividing hematopoietic stem cells in the bone marrow are the major damaged cells by irradiation, the effects of PG101 were first tested on the number of the CFU in the bone marrow cell population. Bone marrow cells were isolated on Days 8, 16, and 24 after irradiation, and were seeded at an average of 10^5 cells per plate in the methylcellulose assay system. In the control-irradiated mice gavaged with PBS, almost no CFCs were formed by Day 8, and the number of CFCs remained very low until Day 24. In contrast, in bone marrow cells from PG101-treated mice, the number of CFCs was already close to the level seen in nonirradiated mice at Day 8, and continued to show such levels during the 24-day period. G-CSF-treated mice also produced the normal number of CFCs (Fig. 1A).

To determine which CFU were affected by PG101, BFU-E and CFU-GM were determined by morphological characteristics. In PBS-treated control mice, neither CFU-GM nor BFU-E were formed properly and their numbers remained significantly lower than nonirradiated mice. In contrast, the number of CFU-GM in PG101-treated mice was comparable with that in nonirradiated normal mice at all time points (Fig. 1B). Consistent with other investigators' findings, repeated injections of G-CSF resulted in an abnormally high number of CFU-GM compared with that of nonirradiated normal mice. The number of BFU-E in PG101-treated mice at Day 8 was approximately one-half of that in nonirradiated mice, but it reached almost the normal level at Day 24 (Fig. 1C). These results suggested that the

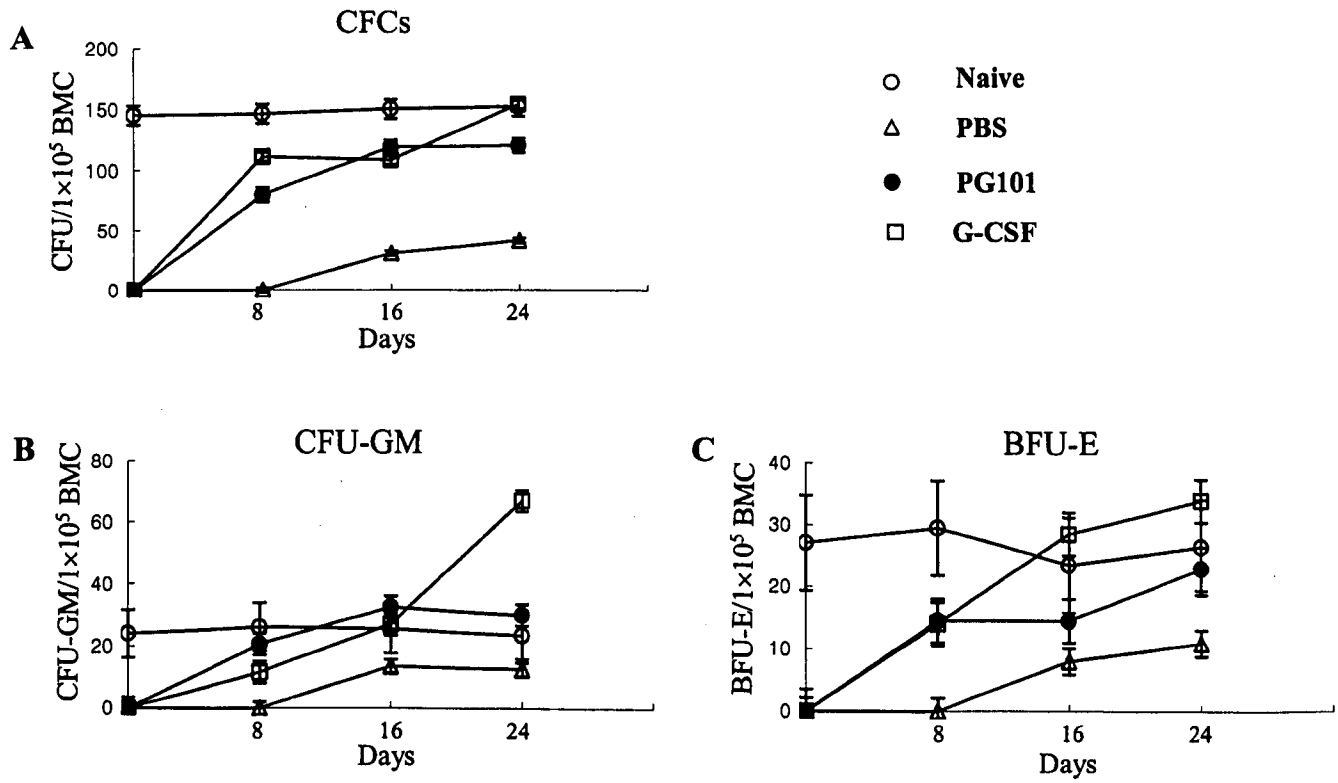

Figure 1. Effects of PG101 on colony-forming units in irradiated mice. Bone marrow cells (1 × 10^5/well) from PBS-, PG101-, or G-CSF-administrated mice were plated on methylcellulose containing growth factors on Days 8, 16, and 24 after irradiation. After 10 days, the total colony number was counted. Colony type was classified by morphological observation. Data are expressed as means ± SEM from three independent experiments.

treatment of PG101 could induce the formation of various early progenitor cells, including those for granulocytes and macrophages.

Cell Types Affected by PG101. To test which cell types are affected by PG101, bone marrow cells were analyzed at Day 24 by flow cytometric analysis using the two antibodies to ER-MP12 and ER-MP20. ER-MP12 interacts with CD31/PECAM-1 on the cell surface, which are expressed at the early stages of hematopoiesis in the progenitor cells, but absent during the later stages of differentiation (16). ER-MP20 binds to the Ly-6C protein that is expressed during the differentiation of the colony forming cells to granulocytes or monocytes (17, 18). The relative presence of these proteins has been used to differentiate six different subpopulations in the bone marrow (19), as shown in Figure 2A. For convenience, each subpopulation is named subset 1 to 6. By a two-color flow cytometric analysis, changes in the bone marrow subpopulations were analyzed after treatment with PG101. As controls, bone marrow cells from irradiated mice treated with PBS or G-CSF were also used. The typical double-staining pattern of bone marrow cells in the normal mouse is shown in Figure 2A. When irradiated mice were treated with PBS, the number of cells in subset 3 was significantly increased (Fig. 2B). This subset is thought to consist mainly of erythroid cells and dead cells. The seemingly increased number of cells in subset 3 probably resulted from the increased number of damaged cells. When mice were administered PG101, the number of cells in subsets 4 and 5 (ER-MP12^{+}20^{+} and ER-MP12^{-}20^{med}) were noticeably increased and became comparable with that in normal mice (Fig. 2C). Similar observations were made in G-CSF-treated animals (Fig. 2D). These results suggested that PG101 might target granulocytes and/or early myeloid progenitor cells.

Effects of PG101 on Differentiation and Proliferation of Granulocytes. Based on the above results, we also tested the effects of PG101 on the differentiation and proliferation of bone marrow cells of the granulocyte lineage by FACS using the two antibodies to c-Kit and Gr-1. c-Kit is the receptor to the stem cell factor. When stem cells are differentiated to the progenitor cells of the next stages, the level of the c-Kit protein is one of the first changes that occur, shifting from low to high (20). However, when cells mature further to lineage-committed cells, c-Kit expression is known to decrease (21). We also examined the Gr-1 protein, which is absent from primitive progenitor cells, but is highly expressed when progenitor cells are committed to the macrophage or granulocyte lineage (22). Cells were analyzed by a single-color flow cytometric analysis using antibodies to c-Kit or Gr-1.

The percentage of c-Kit-positive cells (from femur bone marrow, which accounts for 17.8% of total bone marrow) in irradiated mice was at 19.52% on Day 16 (Fig. 3A, number 6). In PG101-treated mice, the number of c-Kit-positive cells was higher at 24.87%, suggesting a stimulatory effect of PG101 on the development of c-Kit-positive

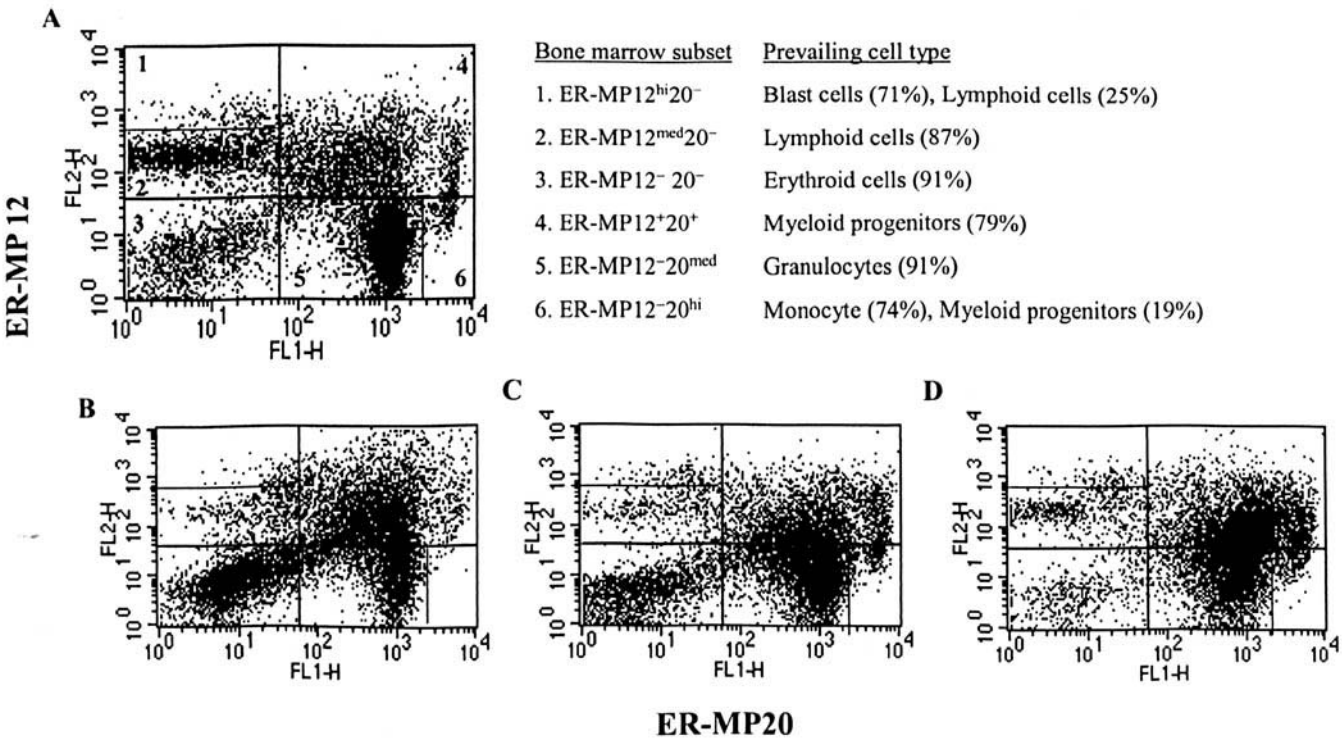

Figure 2. Repopulation of bone marrow cells by PG101 in irradiated mice. Bone marrow cells from PG101-administrated mice were double stained with anti-ER-MP12 and anti-ER-MP20 and bone marrow subsets were analyzed by flow cytometry. Gates for subsets were set on the basis of ER-MP12/20 expression in nonirradiated mice (A). The population in the distinct subsets was determined in irradiated control (B), PG101 (C), and G-CSF (D) at Day 24 after irradiation.

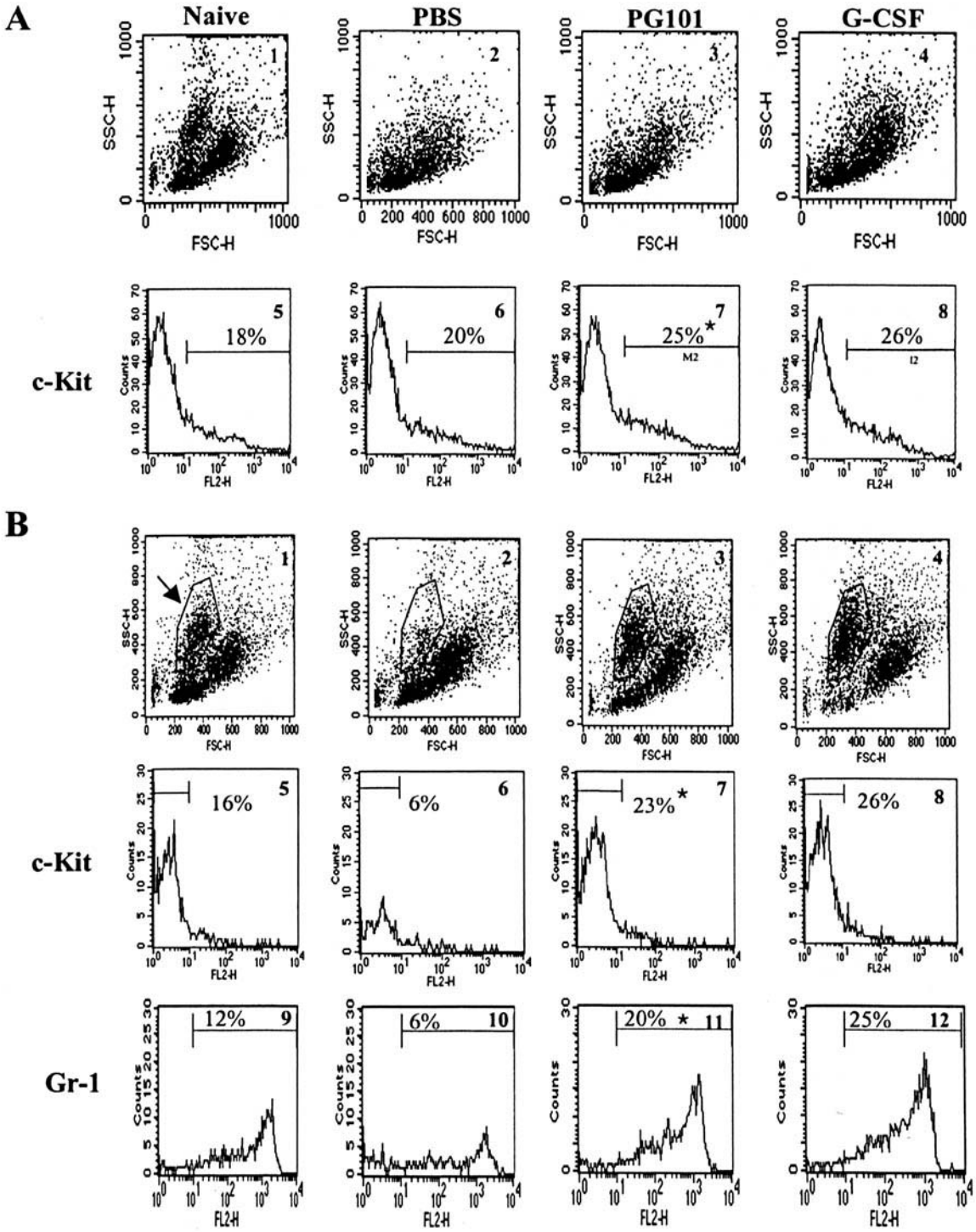

Figure 3. Effects of PG101 on the cells committed to granulocyte in bone marrow in irradiated mice. Bone marrow cells from PBS-, PG101-, and G-CSF-treated mice were stained with PE-conjugated anti-c-Kit antibody or PE-conjugated anti-Gr-1 antibody, respectively, and analyzed by flow cytometry. The expression of c-Kit in bone marrow cells on Day 16 was represented by use of a histogram (A, 5-8). Gate was set on the region representing the increased cell population upon sample treatment in the dot blot (B, 1-4), and the intensity of fluorescence represented in the histogram for c-Kit and Gr-1 (B, 5-12). Percentages are the mean of three mice on Day 16 and 24. $*P < 0.05$ vs control-irradiated mice treated with PBS (Student's *t* test)

progenitor cells (Fig. 3A, Number 7). A similar observation was made in G-CSF-treated mice. It is worth noting that the 25% increase of c-Kit-positive cells in PG 101-treated mice as compared with control mice is very significant because the percentage of c-Kit-positive cells is usually maintained at a low level in bone marrow.

On Day 24, no further increase of c-Kit expression was detectable in total bone marrow cells (data not shown). Most

interestingly, in PG101-treated mice, a cell population not previously present in irradiated mice, but observed in non-irradiated mice, emerged on Day 24, which was not found on Day 16 (compare Fig. 3A, Number 3 with 3B, Number 3). This made the overall pattern of dot plots very similar to that of nonirradiated mice (compare Fig. 3B, Number 1 and 3B, Number 3). This cell population was gated and their fluorescence pattern was further analyzed. Cells were essentially negative to c-Kit, whereas surface Gr-1 was highly expressed, suggesting an increase in the number of mature granulocytes (Fig. 3B, Numbers 7 and 11). The number of these c-Kit-negative and Gr-1-positive cells was very comparable with that in nonirradiated mice. A similar observation was made in G-CSF-treated animals (Fig. 3B, Numbers 8 and 12). In irradiated mice treated with PBS, the number of both c-Kit-negative and Gr-1-positive was very low (Fig. 3B, Numbers 6 and 10). These results indicated that PG101 might be involved in efficiently inducing the differentiation of progenitors and further proliferation of the cells committed to granulocyte lineage.

Effect of PG101 on the Serum Levels of Cytokines. It was previously reported that IL-1β and IL-6 might be involved in the recovery of blood cells after irradiation (23, 24), wheras GM-CSF might play important roles in proliferation and differentiation of bone marrow cells (25). The effects of PG101 on these cytokines were tested in irradiated mice. Levels of the above cytokines in the serum were measured using the commercially available kits at Days 8, 16, and 24. As clearly shown in Figure 4, levels of IL-1β, IL-6, and GM-CSF were significantly increased in PG101-treated mice. In contrast, the level of TNF-α, whose level had been increased after irradiation, was decreased over time. These results suggested that PG101 could increase serum levels of radioprotective cytokines, while decreasing the level of radioinduced TNF-α.

Discussion

Our results suggested that PG101 is a very effective BRM that has significant biological effects on the bone marrow. PG101 helped irradiated mice to rapidly recover the cells that are capable of forming a colony. The daily administration of PG101 for 24 days increased the number of CFU to an almost normal level. PG101 appeared to affect specific target cells. The FACS analysis showed that PG101 increased the number of ER-MP12^{+}20^{+} and ER-MP12^{-} 20^{med} cells, which consist mainly of myeloid progenitor cells and granulocytes. FACS analysis of cells by antibodies to c-Kit and Gr-1 indicated that PG101 induces differentia-

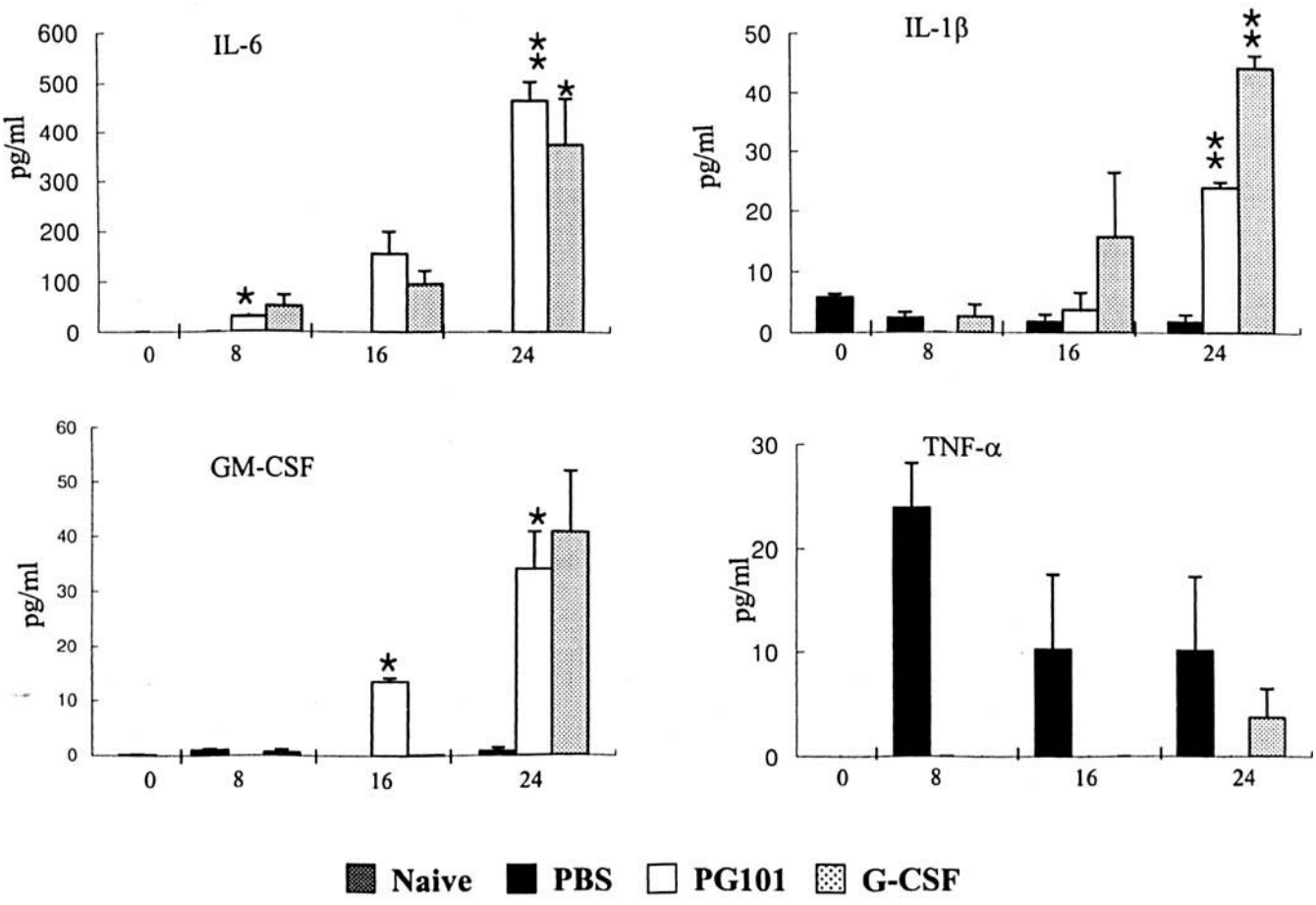

Figure 4. Effects of PG101 on cytokine production in serum in irradiated mice. Serum levels of GM-CSF, IL-1β, IL-6, and TNF-α on Days 8, 16, and 24 were analyzed using ELISAs for control-, PG101-, and G-CSF-treated mice. Data are the expressed as means ± SEM of three mice. $^{*}P < 0.05$; $^{**}P < 0.01$ vs control-irradiated mice treated with PBS (Student's *t* test)

tion of progenitor cells and/or proliferation of the cells committed to granulocytes.

Among cells of granulocyte lineage, neutrophils are one of the most important indicators for assessing the immune capability of patients against infection. If the number of neutrophil reaches less than 500/μl, patients become prone to various fatal infections. Our data suggested that cells of the granulocyte lineage are one of the major target cells by PG101. PG101 increased the number of CFU-GM as early as Day 8. Indeed, our preliminary experiments indicated that peripheral neutrophils are the cell type increased most prominently by treatment with PG101 (data not shown).

Radiation is known to result in serious dysregulation of cytokine expression (26). PG101 increased the levels of IL-1β, IL-6, and GM-CSF over the 24-day period. All these proteins are well known to play important roles in the proliferation and differentiation of various immune cells. TNF-α, which is increased as a consequence of tissue injury and anemia due to radiation (27, 28), is thought to be a key mediator for the pathogenesis of radiation damage (29). It is interesting to find that PG101 significantly reduced the level of TNF- α. All these data suggested that PG101 is able to modulate the dysregulation of cytokine production in radiation damage.

Our results suggest that PG101 is a very potent immune modulator that recovers the radiation-damaged bone marrow system very effectively. Furthermore, PG101 appears to be very safe. During this study, we daily administered 0.5 g/kg over 24 days and found no apparent toxicity, and all visible signs clearly suggested the relative safety of PG101. PG101 is also convenient to use. It is orally administrated, whereas many other reagents that may be used for the same purposes have to be i.v. or s.c. injected. This renders the use of PG101 simple and less invasive. PG101 shows great potential as a supplement or a major therapeutics in immunocompromised or immunosuppressed individuals whose bone marrow system is damaged.

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