

Mitigation of Ionizing Radiation-induced Bone Marrow Suppression by p38 Inhibition and G-CSF Administration

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p38/G-CSF/Radiation/Hematopoietic system.

p38 mitogen-activated protein kinases (p38) has been shown to be activated in hematopoietic stem and progenitor cells after exposure to ionizing radiation (IR) and its activation has been implicated in bone marrow (BM) suppression under various pathological conditions. Therefore, in the present study we investigated whether inhibition of p38 activity alone with SB203580 (SB, a specific p38 inhibitor) or in combination with granulocyte colony-stimulating factor (G-CSF) can mitigate total body irradiation (TBI)-induced BM damage and lethality. Our results showed that p38 inhibition with SB had no significant effect on the 30-day survival rates of the mice exposed to 7.2 Gy TBI when it was used alone but increased the survival of the mice when it was combined with G-CSF. This combined effect may be attributable to a better preservation or stimulation of hematopoietic stem and progenitor cells, because BM cells from SB and G-CSF-treated mice produced more colony forming units-granulocyte-macrophage (CFU-GM) and 4-week cobblestone area forming cells (CAFCs) than the cells from either SB or G-CSF-treated mice after TBI in a colony forming cell assay and a CAFC assay, respectively. These findings suggest that the combined therapy with SB and G-GSF is more effective in mitigating TBI-induced acute BM injury than either agent alone.

INTRODUCTION

Bone marrow (BM) injury is one of the most important side effects of conventional radiotherapy and/or chemotherapy for cancer, which limits the success of cancer treatment and adversely affects quality of cancer patients' life.^{1,2)} In addition, BM suppression is also the primary cause of death after an intentional or unintentional exposure to a lethal dose of total body irradiation (TBI). However, the mechanisms through which ionizing radiation (IR) induces BM injury remains not well understood,³⁾ nor has an effective treatment been developed to mitigate the injury.^{4,5)}

Granulocyte colony-stimulating factor (G-CSF) can mod-

erately reduce the severity and duration of IR- and/or chemotherapy-induced BM injury in experimental animals and cancer patients.^{6,7)} The major effect of G-CSF is restricted to late granulopoietic precursor cells, increasing their proliferation and differentiation. However, such an effect can potentially compromise hematopoietic stem cell (HSC) self-renewal, resulting in long-term myelosuppression.^{8–12)} Thus, it is important to know whether G-CSF can be combined with other drugs or growth factors to achieve better therapeutic efficacy while avoiding its long-term adverse impact on HSCs.^{13–15)}

p38 mitogen-activated protein kinases (p38) belong to a family of serine-threonine protein kinases that are involved in regulation of a variety of biological functions, such as inflammation, cell cycle progression, cell differentiation, cell death and senescence.^{16–18)} In the hematopoietic system, activation of p38 has been implicated in mediating myelosuppression induced by various hematopoietic suppressive cytokines such as IFN- α and - β , TGF- β and TNF- α .¹⁹⁾ Moreover, inhibition of p38 either with pharmacological inhibitors or by genetic approaches have been shown to reduce the severity of acquired aplastic anemia (AA) and myelodysplastic syndromes (MDSs) by inhibiting apoptosis of hematopoietic stem and progenitor cells (HSPCs).^{20,21)}

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Activation of p38 has also been found in HSPCs after ionizing radiation.²² Inhibition of p38 may be a novel therapeutic strategy for mitigating IR-induced BM suppression, particularly in combination with G-CSF. Therefore, in the present study, we examined whether inhibition of p38 with SB203580 (SB, a specific p38 inhibitor) either alone or in combination with G-CSF can mitigate TBI-induced BM injury.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from Vital River (Beijing, China) and housed in the certified animal facility in the Institute of Radiation Medicine, Chinese Academy of Medical Sciences. All mice were used at approximately 8–12 weeks of age. All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Irradiation and treatment

Mice were exposed to γ rays from a ¹³⁷Cs source housed in an Exposure Instrument Gammacell-40 (Atomic Energy of Canada Lim) at a dose-rate of 0.79 Gy per minute. Sham-irradiated animals were treated in a similar manner as irradiated mice except without exposure to IR. After irradiation, animals were returned to the animal facility for daily observation and treatment as described below.

p38 inhibitor SB (SB203580 or 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole, from LC Laboratories, USA) was dissolved in a saline solution containing 30% DMSO. G-CSF (JZJY Cor, China) was diluted to 5 μ g/ml with a saline solution containing 30% DMSO. For SB treatment, mice were given SB at 15 mg/kg via intraperitoneal injection (ip) 24 h after irradiation, and then thereafter every other day for a total of 5 injections. For G-CSF treatment, mice were administered with G-CSF at a dose of 1 μ g/each by ip at 2 h and 6 h after irradiation on the first day, and then twice every day for 5 days. For combination therapy, mice were given both SB and G-CSF as described above. As a control, mice were irradiated and then treated with vehicle in a similar manner as described for SB and/or G-CSF treatments.

BM cell harvesting and cell counting

BM cells were flushed from mouse femur with Hank's balanced salt solution (HBSS) after mice were euthanized. The number of viable BM nucleated cells (BMNCs) was counted using a hemocytometer (Sysmex pocH-100i, Japan) and expressed as $\times 10^6$ /femur.²³

Colony-forming cells (CFC) assay

The CFC assay was performed by culturing BM cells in MethoCult GF M3534 methylcellulose medium (StemCell Technologies, Canada) and the colonies of CFU-granulocyte macrophage (CFU-GM) with more than 30 cells were scored under an invert microscope according to the manufacturer's instruction. The results were expressed as the numbers of CFU-GM ($\times 10^3$) per femur.²⁴

Cobblestone area forming cell (CAFC) assay

CAFC assay is a clonogenic assay, which has been widely used to measure the hematopoietic function of HSCs. BM stromal feeder cells were prepared by seeding 6×10^5 /well BM marrow cells in each well of flat-bottomed 96-well plates. They were exposed to 12 Gy irradiation after 1 week culture. Two weeks later, BM cells harvested from sham-irradiated or irradiated mice were suspended in CAFC medium (Iscove's MDM supplemented with 20% horse serum, 10^{-6} M hydrocortisone, 10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin) and overlaid on the stromal feeder cells in six dilutions and 3-fold apart. Twenty wells were plated for each cell dilution to allow limiting dilution analysis for CAFCs which appeared under the stromal cells layers. Cultures were fed weekly by changing one-half of the medium. Wells were scored positive if at least one phase-dark hematopoietic clone or CAFC (containing 5 or more cells) was seen at 4 weeks after BM cells were seeded. The frequency of CAFC was then calculated by using Poisson statistics as described previously.²⁵ The results from a representative experiment are presented as the mean number of CAFCs per femur \pm SD (n = 3). Similar results were observed in additional experiments.

Statistical analysis

The 30-day survival curves were compared by Kaplan-Meier methods using a log-rank test. The BM cell counts and CFU-GM numbers were compared by one-way ANOVA. *p* value of less than 0.05 was considered significant. Error bars represent \pm SEM.

RESULTS

Thirty-day survival after TBI

To test the effects of SB and/or G-CSF on TBI-induced lethality in mice, we first observed the survival rates of mice after exposure of them to a lethal dose (7.2 Gy) of TBI. As shown in Fig. 1, TBI resulted in 73% mortality in mice treated with vehicle by day 9 after irradiation, and the rest mice died at 12 days post-irradiation. The mortality of the irradiated mice that were treated with SB, G-CSF, and combination of both was 64%, 47%, 40% at day 9 and 93%, 73%, 60% by day 12, respectively. No mice lived more than 13 days after TBI after receiving SB treatment alone. The day 30 survival rates after TBI were 7% and 27% for the mice treated with

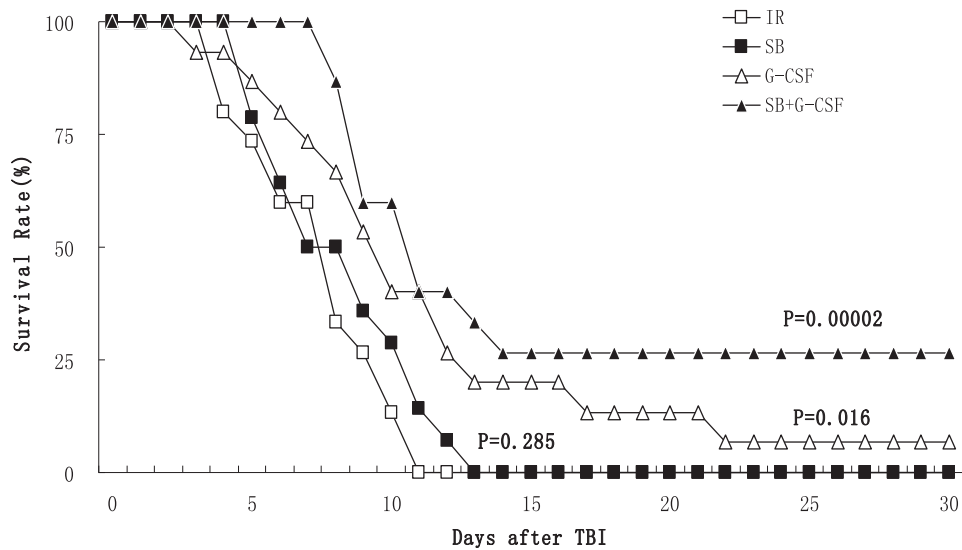


Fig. 1. Effects of SB and/or G-CSF on the survival of mice exposed to TBI (7.2 Gy). After the mice were exposed to 7.2 Gy TBI, they were treated with ip injection of vehicle as irradiation controls (IR, $n = 15$), SB ($n = 14$), G-CSF ($n = 15$), or both ($n = 15$) as described in the Methods. The data is expressed as percentage of surviving mice. The p value shows the difference to the IR group.

G-CSF alone and combination of SB and G-CSF, respectively, which are significantly different from irradiated mice with vehicle treatment ($p < 0.05$). This result suggests that the combination treatment with SB and G-CSF is more effective than either agent alone in mitigating TBI-induced lethality in mice.

Effects of SB and/or G-CSF on BMNC counts after TBI

To determine whether SB and G-CSF reduces TBI-induced lethality is attributable to an increase in hematopoiesis, we analyzed BMNC counts 10 days after mice were exposed to a sub-lethal dose (4 Gy) of TBI. As shown in Fig. 2, BMNC counts ($14.9 \pm 2.2 \times 10^6$ cells/femur) in the irradiated mice receiving vehicle treatment were significantly lower than those in the un-irradiated control group ($18.6 \pm 3.2 \times 10^6$ cells/femur, $p < 0.05$). BMNCs numbers in irradiated mice treated with G-CSF, SB or both (18.1 ± 2.9 , 16.2 ± 2.2 , $19.7 \pm 4.7 \times 10^6$ cells/femur) were greater than vehicle-treated mice after TBI, indicating that treatment with SB and/or G-CSF promoted hematopoiesis after TBI.

Effects of SB and G-CSF on CFU-GM after TBI

To determine whether SB and/or G-CSF increase hematopoiesis after TBI by stimulating hematopoietic progenitor cells, we examined the effects of SB and/or G-CSF on CFU-GM. As shown in Fig. 3, the frequencies (6245 ± 1819 /each femur) of CFU-GM in BM cells from irradiated mice receiving vehicle treatment were significantly lower than those (52537 ± 9864 /each femur) from control un-irradiated mice ($p < 0.01$). The reduction in CFU-GM frequencies was slightly attenuated by the treatment with SB (10251 ± 2874 /

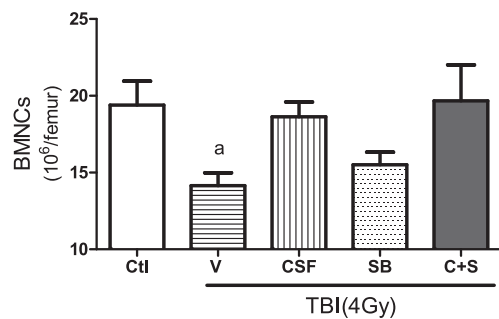


Fig. 2. Effects of SB and/or G-CSF on BMNC counts. After the mice were exposed to 4 Gy TBI, they were treated with ip injection of vehicle (V, $n = 5$), SB ($n = 5$), G-CSF (CSF, $n = 5$), or both (C + S, $n = 5$) as described in the Methods. A group of sham-irradiated control mice was included as a control (Ctl). BMNCs were collected and counted after the mice were euthanized 10 days after TBI. The data are expressed as mean \pm SEM ($n = 5$ for each group). $p < 0.05$ vs. Ctl.

each femur) or G-CSF (12631 ± 2900 /each femur). However, the effect of SB plus G-CSF on CFU-GM frequencies (20975 ± 6445 /each femur) was significantly greater than either agent alone.

Effects of SB and G-CSF on CAFCs after TBI

The effects of SB and/or G-CSF on the hematopoietic function of mouse BM HSCs were analyzed by a CAFC assay. As shown in Fig. 4, the 4-week CAFC frequency in vehicle-treated mice after TBI was lower than that of control un-irradiated mice. The reduction of CAFCs was not affect-

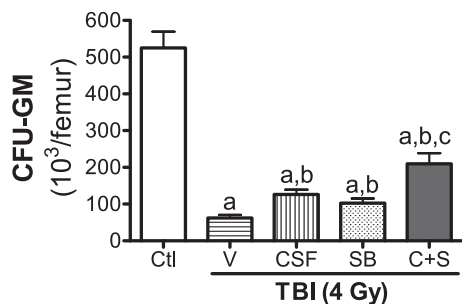


Fig. 3. Effects of SB and/or G-CSF on CFU-GM. After the mice were exposed to 4 Gy TBI, they were treated with ip injection of vehicle (V), SB, G-CSF (CSF), or both (C + S) as described in the Methods. A group of sham-irradiated control mice was included as un-irradiated controls (Ctl). BMNCs were collected and counted after the mice were euthanized 10 days after TBI. They were cultured in MethoCult GF M3534 methylcellulose medium for analysis of CFU-GM. Results are expressed as the number of CFU-GM each femur and presented as mean \pm SEM of three independent experiments. a, $p < 0.05$, vs. Ctl; b, $p < 0.05$, vs. V; and c, $p < 0.05$, vs. SB.

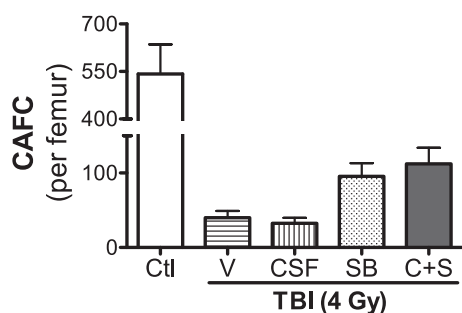


Fig. 4. Effects of SB and/or G-CSF on 4-week CAFCs. After the mice were exposed to 4 Gy TBI, they were treated with ip injection of vehicle (V), SB, G-CSF (CSF), or both (C + S) as described in the Methods. A group of sham-irradiated control mice was included as un-irradiated controls (Ctl). BMNCs were collected and counted after the mice were euthanized 10 days after TBI. HSC function was analyzed by 4-week CAFC assay.

ed by G-CSF treatment, but was attenuated by the treatment with SB or combination of SB and G-CSF. This finding suggests that SB may be more effective in promoting HSC function than G-CSF.

DISCUSSION

Exposure to IR induces various pathophysiological alterations, including BM suppression. Multiple mechanisms, including direct DNA damage and indirect oxidative stress contributed to the tissue injury induced by IR.⁴ Development of an effective treatment to mitigate IR-induced normal tissue injury remains an important area of research in cancer treatment and development of medical countermeasures against nuclear accident and terrorism.^{26–29}

Currently, G-CSF is the only treatment recommended to be given to radiation victims soon after accidental exposure.^{11,30,31} The ASCO Update Committee agreed unanimously that reduction in febrile neutropenia was an important clinical outcome that justifies the use of CSFs, regardless of impact on other factors.³² However, the clinical trials demonstrated G-CSF during radiotherapy could exhaust the bone marrow capacity, probably via promoting HSPCs proliferation and differentiation to negatively affect the self-renewal of HSCs.^{33–35} Therefore, new therapeutic strategies are urgently needed to avoid the negative effect of G-CSF on HSPCs.

An increasing body of evidence from a number of studies carried out during the past few years demonstrates that p38 plays an important role in regulation of the hematopoietic function of HSPCs and its over-activation mediates BM suppression in various pathological conditions.^{19,36} These findings promoted us to examine if p38 inhibition with SB can be exploited as a new strategy to mitigate IR-induced BM suppression either alone or in combination with G-CSF. The results from our study demonstrated that SB alone was not effective in mitigating TBI-induced lethality in mice, but when combined with G-CSF, it enhanced the effect of G-CSF in reducing TBI-induced mortality. This additive or cooperative effect may be attributable to an increase in hematopoiesis, because the number of BMNCs was greater in SB and G-CSF-treated mice after TBI than in irradiated mice receiving either agent treatment. The increase in hematopoiesis is likely resulted from a better preservation or stimulation of HSPCs by p38 inhibition and G-CSF, as BM cells from SB and G-CSF-treated mice after TBI produced more CFU-GM and 4-week CAFCs than the cells from irradiated mice receiving either agent treatment. Therefore, these findings suggest that inhibition of p38 combined with G-CSF treatment can be explored as a more effective medical countermeasure against IR-induced BM toxicity than G-CSF alone.

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