

Inhibiting TGF β 1 has a protective effect on mouse bone marrow suppression following ionizing radiation exposure *in vitro*

Heng ZHANG^{1,2}, Ying-ai WANG³, Aimin MENG², Hao YAN¹, Xinzhuo WANG¹, Jingxiu NIU¹, Jin LI² and Hui WANG^{1,*}

¹Department of Radiation Oncology, Tianjin Union Medical Center, No.190 Jieyuan Road, Nankai District, Tianjin, China

²Institute of Radiation Medicine, Peking Union Medical College (PUMC), No. 238 Baidi Road, Nankai District, Tianjin, China

³Department of Internal medicine, Tianjin Medical University, No. 22 Qixiangtai Road, Hexi District, Tianjin, China

*Corresponding author. Tel: +86 13602018589; Fax: +86-22-87721989; E-mail: ezxwanghui@126.com

(Received 2 October 2012; revised 1 December 2012; accepted 27 December 2012)

Ionizing radiation (IR) causes not only acute tissue damage but also residual bone marrow (BM) suppression. The induction of residual BM injury is primarily attributable to the induction of reactive oxygen species (ROS) pressure in hematopoietic cells. In this study, we examined if SB431542, a transforming growth factor β 1 (TGF β 1) inhibitor, can mitigate IR-induced BM suppression *in vitro*. Our results showed that treatment with SB431542 protected mice bone marrow mononuclear cells (BMMNCs), hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs) from IR-induced suppression using cell viability assays, clonogenic assays and competitive repopulation assays. Moreover, expression of gene-related ROS production in hematopoietic cells was analyzed. The expression of NOX1, NOX2 and NOX4 was increased in irradiated BMMNCs, and that of NOX2 and NOX4 was reduced by SB431542 treatment. Therefore, the results from this study suggest that SB431542, a TGF β 1 inhibitor, alleviates IR-induced BM suppression at least in part via inhibiting IR-induced NOX2 and NOX4 expression.

Keywords: TGF β 1; ionizing radiation; bone marrow suppression; NADPH oxidase

INTRODUCTION

Bone marrow (BM) suppression is the most common dose-limiting side-effect of conventional cancer therapy using ionizing radiation (IR) [1, 2] and is the primary cause of death after accidental exposure to a high dose of IR. The myelosuppression not only worsens the outcome of cancer treatment but also adversely affects the quality of life of cancer patients. However, the mechanisms through which ionizing radiation (IR) induces BM injury are not well understood, nor has an effective treatment been developed to mitigate the injury.

Transforming growth factor β 1 (TGF β 1) is a pleiotropic cytokine involved in many hematopoietic cells activities, including growth, differentiation, quiescence, apoptosis and mobilization [3, 4]. TGF β 1 is secreted as a latent precursor molecule (LTGF β) bound to LTGF β -binding protein, LTBP [5]. LTGF β can be activated by IR in the extracellular space of irradiated tissue as well as proteolytic processes [6, 7]. Activated TGF- β 1 can activate TGF- β type I and type II

receptors, which are responsible for activating intracellular mediators such as Smad proteins and p38 mitogen-activated protein kinase (MAPK) [8, 9].

Previous work has shown that IR-induced oxidative stress in hematopoietic cells via the NADPH oxidase (NOX) pathway at least partly contributes to IR-induced BM failure [10]. Mitochondria have been widely accepted to be the main source of cellular-derived reactive oxygen species (ROS). However, recent studies demonstrate that cells can also produce ROS deliberately through a family of tightly regulated NOXs that are homologues of the phagocyte oxidase [11, 12]. In lung fibroblasts, TGF β -induced NADH oxidase production of hydrogen peroxide begins 8 h after TGF β stimulation with peak activity at 16 h [13]. In fetal rat hepatocytes, it is demonstrated that TGF β -induced ROS occurred 4 h after TGF β treatment [14]. In addition, it was proved TGF β also plays core roles in up-regulation of NOXs in hepatocytes, kidney myofibroblasts, aortic smooth muscle cells, etc. at least *in vitro* [15–18].

The aim of the present work was to analyze whether IR-induced BM suppression could be ameliorated by inhibiting TGF β 1, and to study the role of NOXs in this.

MATERIALS AND METHODS

Reagents

Anti-Mouse-CD45.1-percp-cy5.5 (clone A20, Ly5.1), Anti-Mouse-CD45.2-PE (clone 104, Ly5.2), biotin-conjugated anti-Mouse-CD4 (clone GK1.5), anti-Mouse-CD45R/B220 (clone RA3-6B2), anti-Mouse-Ly6G/Gr-1 (clone RB6-8C5), anti-Mouse-CD11b (clone M1/70) and APC-Cy7-conjugated streptavidin were obtained from eBioscience (San Diego, CA, USA). SB431542 was obtained from Sigma (St. Louis, MO, USA).

Mice

Male C57BL/6 mice were purchased from the Institute of Laboratory Animal Sciences (PUMC, Beijing, China) and housed five to a cage at an Animal Care-certified animal facility in the Institute of Radiation Medicine, PUMC. They received food and water *ad libitum*. All of the mice were used at approximately 8–10 weeks of age. The Institutional Animal Care and Use Committee of PUMC approved all experimental procedures used in this study.

Treatment of BMMNCs with IR and SB431542

The femora and tibiae were harvested from the mice immediately after they were euthanized with CO₂. BMMNCs, isolated as described previously [1, 2] and incubated (1×10^6 /ml in complete medium) with SB431542 (1×10^{-3} M – 1×10^{-9} M) or with 0.2% dimethylsulfoxide (DMSO) (vehicle control) at 37°C for 60 min. Then, cells were exposed to 0, 1, 2 or 4 Gy irradiation generated in an Exposure Instrument Cammacell-40 ¹³⁷Cesium-irradiator (Atomic Energy, Lin, CA, USA) at a rate of 0.76 Gy/min. Cells were incubated at 37°C, with 5% CO₂ and 100% humidity for various times as indicated in individual experiments.

Cell viability assays

The cells (1×10^5 cells/well in 100 μ l medium) were plated in a 96-well plate and were cultured for 18 h. Cell viability was monitored using the luminescent-based CellTiter Glo TM (Promega, Madison, WI, USA) according to the manufacturer's recommended protocols [19]. Luminescence of each well was read using the Infinite M200 multimode microplate reader (TECAN, Switzerland). Cell viability was normalized as a percentage of the untreated cells. Each experiment was done in triplicate and the results were averaged.

Clonogenic assays

CFU-GM was analyzed using MethoCult M3534 medium (Stem Cell Technologies, Vancouver, BC, Canada). BM-MNCs incubated with SB431542 and irradiated as

described above were suspended in Methocult M3534 medium at 2×10^4 or 1×10^5 viable cells/ml and seeded in wells of 24-well plates. The plates were incubated for 7 days. Colonies of ≥ 50 cells were scored under an inverted microscope on Day 7, and results were expressed as the number of CFU-GM per 10^5 cells.

Competitive repopulation assay (CRA)

Competitive repopulation assays were performed using the Ly5 congenic mouse system as described previously [10, 20]. After incubation with SB431542 (1 μ M) or exposure to irradiation (2 Gy) as described above, donor cells (C57BL/6-Ly-5.1 mice, 1×10^5 BMMNCs) were mixed with 1×10^5 competitive BM-MNCs pooled from three C57BL/6-Ly5.1/Ly5.2 hybrid mice. Cells were transplanted into lethally irradiated (9.0 Gy IR) C57BL/6-Ly-5.2 mice (seven recipients/group) by lateral canthus-vein injection. For analysis of engraftment, peripheral blood was obtained from the medial canthus using heparin-coated micropipettes (Drummond Scientific, Broomall, PA, USA) at 2 months after transplantation from all the recipients. After red blood cells had been lysed with 0.15 M NH₄Cl solution, the blood samples were stained with FITC-conjugated anti-CD45.1, PE-conjugated anti-CD45.2, PerCP-conjugated anti-B220, APC-conjugated anti-CD3, PE/Cy7-conjugated Anti-Gr-1 and CD11b, and were analyzed with a LSR II flow cytometer (BD Bioscience, San Jose, CA, USA).

Quantification Real-time PCR assays

BM-MNCs were incubated with SB431542 or exposed to irradiation (2 Gy) as described above and cells were incubated for 4 or 24 h. Total RNA was extracted from the BMMNCs using TRIzol reagent (Applied Biosystems, Grand Island, NY, USA) following the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using RNA PCR Kit (AWV) Ver. 3.0 (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. PCR primers for the T β R2, NOX1, NOX2, NOX4 and the housekeeping gene GAPDH were obtained from Sangon Biotech (Shanghai, China). The sequences of primers used in this study were: T β R2, 5'-TAC TCT GGA GAC GGT TTG-3' (forward) and 5'-TGC TGG TGG TGT ATT CTT-3' (reverse); NOX1, 5'-TCG ACA CAC AGG AAT CAG GA-3' (forward) and 5'-TTA CAC GAG AGA AAT TCT TGG G-3' (reverse); NOX2, 5'-TGC AGT GCT ATC ATC CAA GC-3' (forward) and 5'-CTT TCT CAG GGG TTC CAG TG-3' (reverse); NOX4, 5'-GAT TTC TGG ACC TTT GTG CCT TT-3' (forward) and 5'-TGA TGG TGA CAG GTT TGT TGC T-3' (reverse); GAPDH, 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3' (forward) and 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3' (reverse). cDNA samples were mixed with primers and SYBR Master Mix (ABI Bioscience Inc.) in a total volume of 25 μ l. All samples were analyzed in triplicate

using an ABI Prism 7500 Sequence Detection System (Applied Biosystems-Life Technologies). Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The threshold cycle (CT) values for each reaction were determined and averaged using TaqMan SDS analysis software (Applied Biosystems-Life Technologies). The changes in target genes expression were calculated using the comparative CT method (fold changes = $2^{[-\Delta\Delta CT]}$) as described previously [21].

Western blot assays

BM-MNCs were incubated with SB431542 or exposed to irradiation (2 Gy) as described above and cells were incubated for 24 h. Harvested cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). The protein concentration was estimated using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Cell lysates (50 μ g) were loaded onto 5–10% gradient polyacrylamide gels. Proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore, MA, USA) and immunolabeled using 1:400 dilutions of antibodies against TBR1, NOX2, NOX4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 1:2000 dilutions of antibodies against β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The enhanced chemiluminescence plus reagent (Boster Biotechnology Co., Wuhan, China) was used for chemiluminescent signal detection. Quantitative analysis was carried out using Quantity One version 4.6.2 (<http://www.bio-rad.com>).

Statistical analysis

The data were analyzed by analysis of variance (ANOVA). In the event that ANOVA justified post hoc comparisons between group means, these were conducted using the Student–Newman–Keuls test for multiple comparisons. For experiments in which only single experimental and control groups were used, group differences were examined using the unpaired Student's *t* test. Differences were considered significant at $P < 0.05$. All of these analyses were done using SPSS 16.0 software (SPSS Inc.).

RESULTS

SB431542 protected BMMNCs from irradiation injury *in vitro*

Luminescence assays were performed to evaluate cell viability which manifested drugs' radiation protective effect on BMMNCs. Fig. 1A depicts the SB431542 toxicity, in which SB431542 at concentrations higher than 10 μ M inhibited the bone marrow cell viability. Therefore, SB431542 at concentrations lower than 10 μ M was chosen

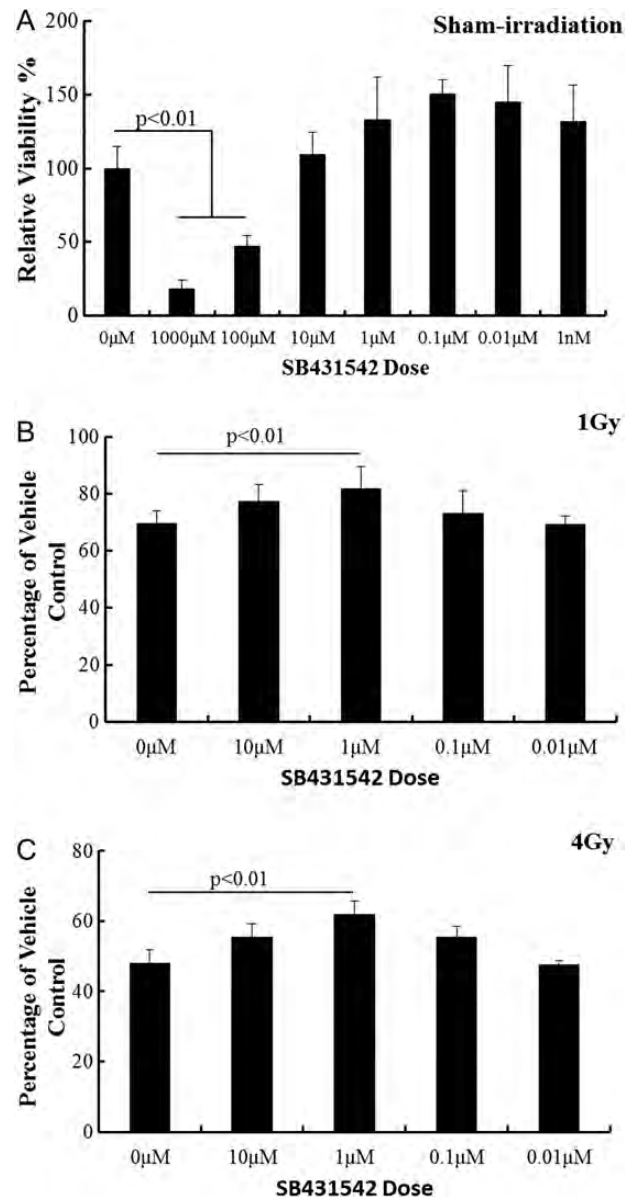


Fig. 1. SB431542 reduced IR-induced suppression of BMMNC viability. The cells were sham-irradiated as a control or sublethally irradiated with 1–4 Gy IR after receiving vehicle or SB431542 treatment and were cultured for 18 h. Cell viability was monitored as described in the text. (A) Cell toxicity assays; (B) cells treated with 1 Gy irradiation; (C) cells treated with 4 Gy irradiation. Data were expressed as relative viability as mean \pm SE ($n = 6$).

for the subsequent experiments. As shown in Fig. 1B and C, compared with the sham irradiation group, BMMNC's viabilities decreased significantly after irradiation exposure (1 Gy for 30.28%, $P < 0.01$; 4 Gy for 51.94%, $P < 0.01$). Treatment with SB431542 (1 μ M) increased cell viabilities of irradiated BMMNCs (1 Gy for 13.25%, $P < 0.01$; 4 Gy for 20.81%, $P < 0.01$). SB431542 treatment displays a

protective effect on irradiated BMMNCs at 4 Gy better than at 1 Gy.

SB431542 raised the ability of forming colonies of CFU-GM

Clonogenic assays were performed to evaluate viability of HPCs affected by IR and SB431542. As shown in Fig. 2, SB431542 was capable of increasing CFU-GM in sham-irradiated cells, whereas the cells treated with different dose of irradiation (1–4 Gy) exhibited a diminished ability to form CFU-GM by 42.1–89.5% ($P < 0.01$) and treatment with SB431542 (10 μM –0.1 μM) enhanced the ability to form CFU-GM by 60.0–292.4% ($P < 0.01$) significantly. These results suggest SB431542 could ameliorate IR-induced mice HPC injury.

SB431542 enhances long-term and multi-lineage engraftment of irradiated HSCs

Because long-term and multi-lineage engraftment is a gold standard to measure HSC function, we performed a competitive repopulation assay to validate whether IR-induced HSC function decline could be ameliorated by SB431542 treatment. As shown in Fig. 3, mice receiving donor cells

exposed to irradiation with vehicle treatment showed a substantial decrease in donor cell engraftment in all the lineages 2 months after transplantation. Treated with

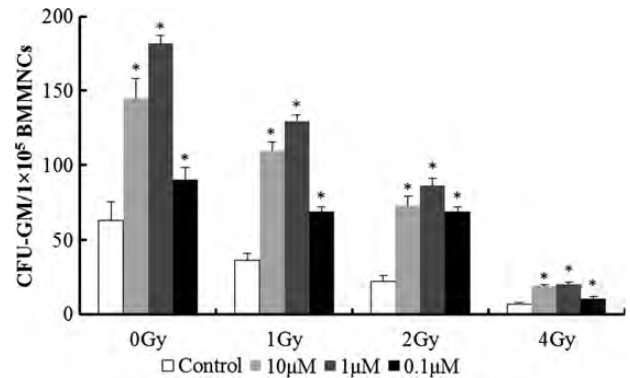


Fig. 2. SB431542 reduces IR-induced suppression of HPC clonogenic function. Mice BMMNCs were sham-irradiated as a control or sublethally irradiated with 1–4 Gy IR after receiving vehicle or SB431542 treatment. Clonogenic function of HPCs in BM-MNCs was analyzed by CFC assay. Colonies of ≥ 50 cells were scored under an inverted microscope on Day 7, and results were expressed as the number of CFU-GM per 10^5 cells. Data are presented as mean \pm SE. * $P < 0.01$ vs. control, $n = 6$.

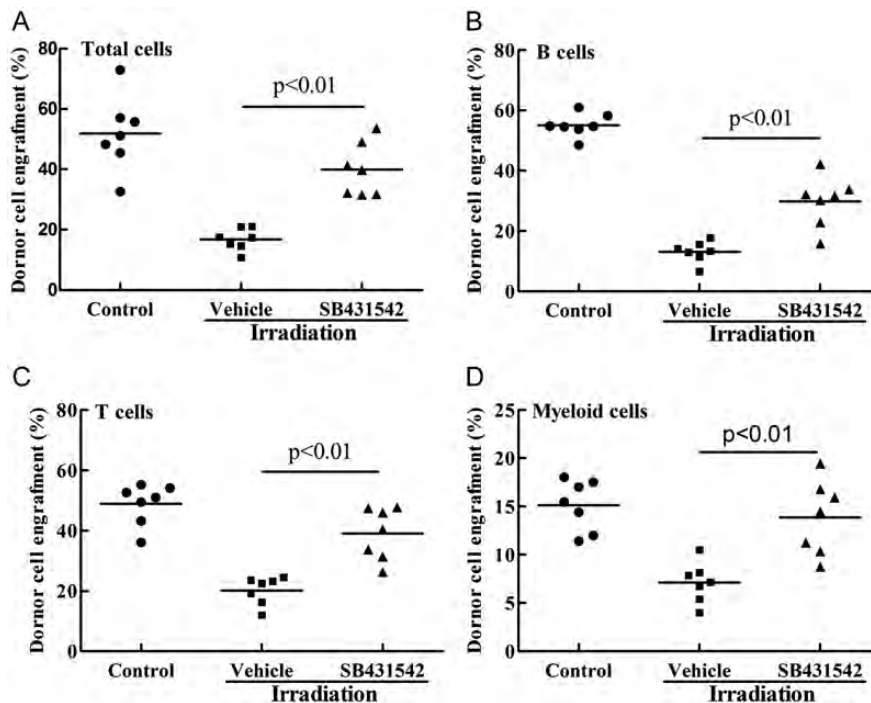


Fig. 3. SB431542 reduces IR-induced suppression of HSC long-term engraftment after transplantation. Donor BMMNCs, treated with IR (2 Gy) after receiving vehicle or SB431542 (1 μM), were mixed with competitive cells. Cells were transplanted into receptor mice as described in the text, and donor cell engraftment was analyzed 2 months after transplantation. The data are expressed as means \pm SE of percentage of donor-derived cells as: (A) leukocytes (CD45.1 + cells), (B) B cells (CD45.1 + B220 + cells), (C) T cells (CD45.1 + CD3 + cells) and (D) myeloid cells (CD45.1 + CD11b + and/or Gr-1 + granulocyte–monocyte–macrophage) in the peripheral blood ($n = 7$ recipient mice/group).

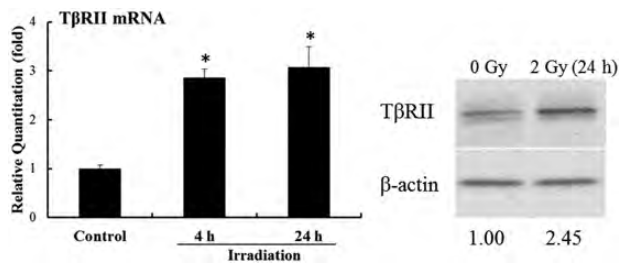


Fig. 4. IR induces expression of TβRII in BMMNCs. The cells were sham-irradiated as a control or irradiated with 2.0 Gy IR. They were incubated for 4–24 h and analyzed for the mRNA and protein expression of TβRII by qRT-PCR and western blot assays. The levels of TβRII mRNA expression are expressed as means ± SE of fold changes compared with the control ($n = 3$). * $P < 0.05$ vs. control.

SB431542 (1 μM), the donor cell engraftment increased 23.1% at 2 months, 16.7% of B cells, 18.8% of T cells and 6.7% of myeloid cells derived from donor cells. These findings indicate that SB431542 treatment indeed preserved the function of HSCs after irradiation exposure, resulting in enhanced long-term and multi-lineage engraftment after BM transplantation.

Irradiation increased TβRII expression in BMMNCs

TGFβ1 can be activated by ionizing irradiation in the extracellular space of irradiated tissue as well as proteolytic processes [6, 7]. To confirm irradiation exposure could up-regulate TGFβ1 activity in bone marrow hematopoietic cells, TβRII expression in BMMNCs exposed to irradiation was analyzed. As shown in Fig. 4, after irradiation exposure, TβRII mRNA and protein expression were up-regulated. These results suggest irradiation might activate the TGFβ pathway partly by over-expression of TβRII.

SB431542 induced down-regulation of NOX expression

Previous work has shown that IR-induced ROS stress in hematopoietic cells via up-regulating NOX4 at least partly contributes to IR-induced BM failure [10]. In this study, NOX1, NOX2 and NOX4 expression in BMMNCs treated with irradiation and SB431542 was investigated. As shown in Fig. 5, irradiation exposure up-regulated NOX1 mRNA expression significantly by 4.07-fold ($P < 0.01$) at 4 h, and by 1.61-fold ($P < 0.05$) at 24 h. After irradiation, NOX2 mRNA expression was up-regulated by irradiation (1.56-fold, 4 h, $P < 0.01$; 1.26-fold, 24 h, $P < 0.05$). Compared with sham-irradiated cells, a consistently higher level (about 2-fold) of NOX4 mRNA expression of irradiated cells was observed from 4–24 h. Treated with SB431542 (1 μM), NOX2 and NOX4 mRNA expression was down-regulated, but not NOX1. These data, verified by western

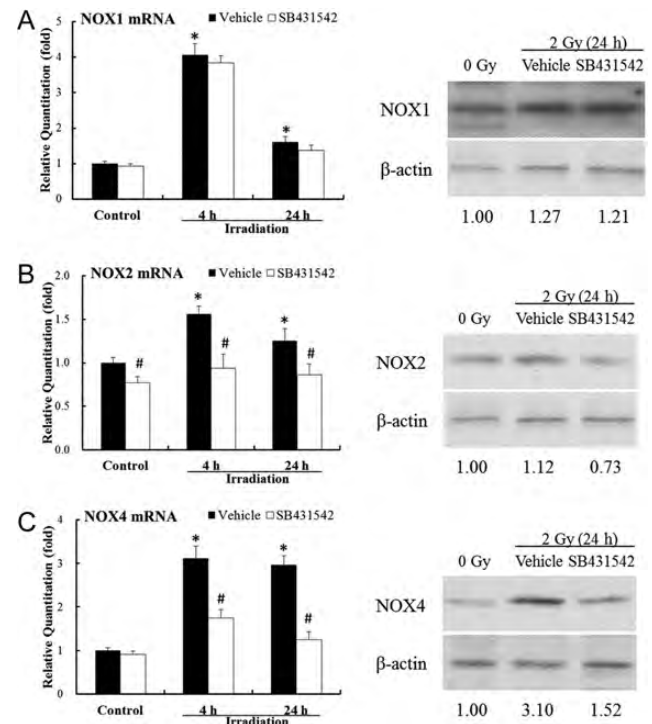


Fig. 5. SB431542 inhibits IR-induced expression of NOX2 and NOX4 in BMMNCs. The cells were sham-irradiated as a control or irradiated with 2.0 Gy IR after receiving vehicle or SB431542 (1 μM) treatment. They were incubated for 4–24 h and analyzed for the mRNA and protein expression of NOX1, NOX2 and NOX4 using qRT-PCR and western blot assays. (A) The levels of NOX1 mRNA and protein expression; (B) the levels of NOX2 mRNA and protein expression; (C) the levels of NOX4 mRNA and protein expression. Results of mRNA expression are expressed as means ± SE of fold changes compared with those of the control ($n = 3$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. vehicle.

blots assays as shown in Fig. 5, suggest radiation exposure up-regulates NOX expression in BMMNCs, which at least partly contributes to oxidative stress in the BM hematopoietic system. And SB431542, a TGFβ1 inhibitor, could reduce IR-induced up-regulation of NOX2 and NOX4 expression.

DISCUSSION

In spite of the extensive study of TGFβ1 inhibitor to prevent fibroblast activity [22, 23], the pharmacological effect of SB431542 on IR-induced BM suppression has not been well investigated. In this study, we examined whether SB431542 can mitigate IR-induced BM injury *in vitro*. Our results showed that IR increased TβRII mRNA expression in BMMNCs, which provides additional evidence that IR could up-regulate TGFβ1 activity. Then, it was found that treatment with SB431542 significantly alleviated depressed

clonogenic function of HPCs and HSCs induced by IR. It was also observed that expression of NOX1, NOX2 and NOX4 in BMMNCs was up-regulated by IR and that of NOX2 and NOX4 was ameliorated by treatment with SB431542. Therefore, results from this study demonstrate that SB431542, a widely used TGF β 1 inhibitor, has the potential to be used as a therapeutic agent to mitigate IR-induced BM suppression in part via inhibition of NOX expression.

Increasing evidence suggests that IR-induced injury is caused, at least in part, by induction of oxidative stress. This suggestion is in agreement with our previous findings [1, 10], which demonstrate IR-induced up-regulation of NOXs in hematopoietic cells at least partly contribute to IR-induced BM failure [10], and using antioxidants such as N-acetylcysteine (NAC), Mn(III) meso-tetrakis-(N-ethylpyridinium-2-yl) porphyrin (MnTE) and resveratrol effectively ameliorates IR-induced BM failure in mice [10, 20, 24]. Elegant work by Bondi *et al.* [17] showed that TGF β 1 increases both the activity of NADPH oxidase and expression of NOX2 and NOX4 in kidney, indicating that this growth factor induces production of ROS, which could be effectively decreased by inhibiting T β RI. It has also been reported that treatment with TGF β 1 increases NOX4 expression and activity in human aortic smooth muscle cells [13], pulmonary fibroblasts [25], airway smooth muscle [26] and in hepatocytes [27]. Our work showed that IR increased NOX1, NOX2 and NOX4 expression in mice BMMNCs, and treatment with SB431542 decreased NOX2 and NOX4 expression after IR. These data suggest inhibiting TGF β 1 could attenuate IR-induced ROS stress via the NOX pathway, which at least partly protects mouse hematopoietic function from IR damage. However, SB431542 functions only as a radiation protectant to reduce IR-induced BM failure, because in our preliminary cell viability assays, we found that post-IR treatment with SB431542 had no significant effect on the activity of the BMMNCs exposed to IR.

Radiation-induced fibrosis, an important side-effect in the treatment of cancer, plays a core role in injury of normal tissues or organs, which not only adversely affects the quality of life of cancer patients but also worsens the outcome of cancer treatment [23]. Increasing evidence shows that TGF β 1 is the major cytokine responsible for the regulation of fibroblast proliferation and differentiation [28, 29]. IR not only induces long-term TGF β 1 overexpression owing in part to oxidative stress and an inflammatory response, but also activates LTGF β in the extracellular space of irradiated tissue as well as proteolytic processes [6, 7]. Many studies suggest inhibiting TGF β 1 could protect lungs, kidneys, blood vessels, liver, skin, etc. from radiotherapy-induced injury [30–33]. In addition, TGF- β 1 inhibitor can attenuate tumor cell migration [34] and enhance sensitivity to radiation therapy in pancreatic cancer, glioblastoma and breast cancer

[35–37]. TGF- β 1 antibody was tested in a phase III clinical trial for breast cancers, prostate cancers, colon cancers and renal carcinoma [33]. Therefore, SB431542 has the potential to increase the therapeutic efficacy of radiotherapy not only by reducing normal tissue injury, but also by inhibiting tumor growth.

In summary, our study shows that SB431542 ameliorates IR-induced BM suppression. These findings provide a better understanding that TGF- β 1 inhibitor may have the potential to be used as a therapy for other oxidative stress-related organ injury.

FUNDING

This study was supported by Wu Jieping Medical Foundation (No 320.6750.12342), National Natural Science Foundation of China (No 81129020 and 81072237) & Tianjin Natural Science Foundation (11JCZDJ19100).

REFERENCES

1. Wang Y, Schulte BA, LaRue AC *et al.* Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood* 2006;**107**:358–66.
2. Meng A, Wang Y, Van Zant G *et al.* Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. *Cancer Res* 2003;**63**:5414–19.
3. Ruscetti FW, Akel S, Bartelmez SH. Autocrine transforming growth factor-beta regulation of hematopoiesis: many outcomes that depend on the context. *Oncogene* 2005;**24**: 5751–63.
4. Soderberg SS, Karlsson G, Karlsson S. Complex and context dependent regulation of hematopoiesis by TGF-beta superfamily signaling. *Ann New York Acad Sci* 2009;**1176**:55–69.
5. Munger JS, Harpel JG, Gleizes PE *et al.* Latent transforming growth factor-beta: structural features and mechanisms of activation. *Kidney International* 1997;**51**:1376–82.
6. Jobling MF, Mott JD, Finnegan MT *et al.* Isoform-specific activation of latent transforming growth factor beta (LTGF-beta) by reactive oxygen species. *Radiation Res* 2006;**166**:839–48.
7. Ehrhart EJ, Segarini P, Tsang ML *et al.* Latent transforming growth factor beta1 activation in situ: quantitative and functional evidence after low-dose gamma-irradiation. *FASEB J* 1997;**11**:991–1002.
8. Watanabe H, de Caestecker MP, Yamada Y. Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells. *J Biol Chem* 2001;**276**:14466–73.
9. Yano H, Hamanaka R, Nakamura M *et al.* Smad, but not MAPK, pathway mediates the expression of type I collagen in radiation induced fibrosis. *Biochemical Biophys Res Comm* 2012;**418**:457–63.
10. Wang Y, Liu L, Pazhanisamy SK *et al.* Total body irradiation causes residual bone marrow injury by induction of persistent

- oxidative stress in murine hematopoietic stem cells. *Free Radical Biol Med* 2010;**48**:348–56.
11. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007;**87**:245–313.
 12. Lambeth JD. Nox enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. *Free Radical Biol Med* 2007;**43**:332–47.
 13. Thannickal VJ, Fanburg BL. Activation of an H₂O₂-generating NADH oxidase in human lung fibroblasts by transforming growth factor beta 1. *J Biol Chem* 1995;**270**:30334–8.
 14. Sanchez A, Alvarez AM, Benito M *et al.* Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor beta in fetal rat hepatocytes in primary culture. *Hepatology* 1997;**26**:935–43.
 15. Martin-Garrido A, Brown DI, Lyle AN *et al.* NADPH oxidase 4 mediates TGF-beta-induced smooth muscle alpha-actin via p38MAPK and serum response factor. *Free Radical Biol Med* 2011;**50**:354–62.
 16. Caja L, Sancho P, Bertran E *et al.* Overactivation of the MEK/ERK pathway in liver tumor cells confers resistance to TGF-β-induced cell death through impairing up-regulation of the NADPH oxidase NOX4. *Cancer Res* 2009;**69**:7595–602.
 17. Bondi CD, Manickam N, Lee DY *et al.* NAD(P)H oxidase mediates TGF-beta1-induced activation of kidney myofibroblasts. *J Am Soc Nephrol* 2010;**21**:93–102.
 18. Black D, Lyman S, Qian T *et al.* Transforming growth factor beta mediates hepatocyte apoptosis through Smad3 generation of reactive oxygen species. *Biochimie* 2007;**89**:1464–73.
 19. Noah JW, Severson W, Noah DL *et al.* A cell-based luminescence assay is effective for high-throughput screening of potential influenza antivirals. *Antiviral Res* 2007;**73**:50–9.
 20. Li H, Wang Y, Pazhanisamy SK *et al.* Mn(III) meso-tetrakis-(N-ethylpyridinium-2-yl) porphyrin mitigates total body irradiation-induced long-term bone marrow suppression. *Free Radical Biol Med* 2011;**51**:30–7.
 21. Zhang H, Li J, Wang YY *et al.* Retinoblastoma 94 enhances radiation treatment of esophageal squamous cell carcinoma in vitro and in vivo. *J Radiat Res* 2012;**53**:117–24.
 22. Andarawewa KL, Paupert J, Pal A *et al.* New rationales for using TGFbeta inhibitors in radiotherapy. *Int J Radiat Biol* 2007;**83**:803–11.
 23. Pohlert D, Brenmoehl J, Löffler I *et al.* TGF-beta and fibrosis in different organs – molecular pathway imprints. *Biochim Biophys Acta* 2009;**1792**:746–56.
 24. Zhang H, Zhai Z, Wang Y *et al.* Resveratrol ameliorates ionizing irradiation-induced long-term hematopoietic stem cell injury in mice. *Free Radical Biol Med* 2013;**54**:40–50.
 25. Amara N, Goven D, Prost F *et al.* NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFbeta1-induced fibroblast differentiation into myofibroblasts. *Thorax* 2010;**65**:733–8.
 26. Michaeloudes C, Sukkar MB, Khorasani NM *et al.* TGF-beta regulates Nox4, MnSOD and catalase expression, and IL-6 release in airway smooth muscle cells. *Am J Physiol Lung Cellular Molecular Physiol* 2011;**300**:L295–304.
 27. Carmona-Cuenca I, Roncero C, Sancho P *et al.* Upregulation of the NADPH oxidase NOX4 by TGF-beta in hepatocytes is required for its pro-apoptotic activity. *J Hepatol* 2008;**49**:965–76.
 28. Yano H, Hamanaka R, Nakamura M *et al.* Smad, but not MAPK, pathway mediates the expression of type I collagen in radiation induced fibrosis. *Biochem Biophys Res Comm* 2012;**418**: 457–63.
 29. O'Sullivan B, Levin W. Late radiation-related fibrosis: pathogenesis, manifestations, and current management. *Sem Radiat Oncol* 2003;**13**:274–89.
 30. Reeves A, Zagurovskaya M, Gupta S *et al.* Inhibition of transforming growth factor-beta signaling in normal lung epithelial cells confers resistance to ionizing radiation. *Int J Radiat Oncol Biology Phys* 2007;**68**:187–95.
 31. Scharpfenecker M, Floot B, Russell NS *et al.* Endoglin haploinsufficiency reduces radiation-induced fibrosis and telangiectasia formation in mouse kidneys. *Radiotherapy Oncol* 2009;**92**:484–91.
 32. Liu Y, Kudo K, Abe Y *et al.* Inhibition of transforming growth factor-beta, hypoxia-inducible factor-1alpha and vascular endothelial growth factor reduced late rectal injury induced by irradiation. *J Radiat Res* 2009;**50**:233–9.
 33. Anscher MS. Targeting the TGF-beta1 pathway to prevent normal tissue injury after cancer therapy. *Oncologist* 2010;**15**:350–9.
 34. Jakowlew SB. Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev* 2006;**25**:435–57.
 35. Ahmed MM, Alcock RA, Chendil D *et al.* Restoration of transforming growth factor-beta signaling enhances radiosensitivity by altering the Bcl-2/Bax ratio in the p53 mutant pancreatic cancer cell line MIA PaCa-2. *J Biol Chem* 2002;**277**:2234–46.
 36. Zhang M, Kleber S, Rohrich M *et al.* Blockade of TGF-beta signaling by the TGFbetaR-I kinase inhibitor LY2109761 enhances radiation response and prolongs survival in glioblastoma. *Cancer Res* 2011;**71**:7155–67.
 37. Bouquet F, Pal A, Pilonis KA *et al.* TGFbeta1 inhibition increases the radiosensitivity of breast cancer cells in vitro and promotes tumor control by radiation in vivo. *Clin Cancer Res* 2011;**17**:6754–65.