Whole-Body Proton Irradiation Causes Long-Term Damage to Hematopoietic Stem Cells in Mice

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Space flight poses certain health risks to astronauts, including exposure to space radiation, with protons accounting for more than 80% of deep-space radiation. Proton radiation is also now being used with increasing frequency in the clinical setting to treat cancer. For these reasons, there is an urgent need to better understand the biological effects of proton radiation on the body. Such improved understanding could also lead to more accurate assessment of the potential health risks of proton radiation, as well as the development of improved strategies to prevent and mitigate its adverse effects. Previous studies have shown that exposure to low doses of protons is detrimental to mature leukocyte populations in peripheral blood, however, the underlying mechanisms are not known. Some of these detriments may be attributable to damage to hematopoietic stem cells (HSCs) that have the ability to self-renew, proliferate and differentiate into different lineages of blood cells through hematopoietic progenitor cells (HPCs). The goal of this study was to investigate the long-term effects of low-dose proton irradiation on HSCs. We exposed C57BL/6J mice to 1.0 Gy whole-body proton irradiation (150 MeV) and then studied the effects of proton radiation on HSCs and HPCs in the bone marrow (BM) 22 weeks after the exposure. The results showed that mice exposed to 1.0 Gy whole-body proton irradiation had a significant and persistent reduction of BM HSCs compared to unirradiated controls. In contrast, no significant changes were observed in BM HPCs after proton irradiation. Furthermore, irradiated HSCs and their progeny exhibited a significant impairment in clonogenic function, as revealed by the cobblestone area-forming cell (CAFC) and colony-forming cell assays, respectively. These long-term effects of proton irradiation on HSCs may be attributable to the induction of chronic oxidative stress in HSCs, because HSCs from irradiated mice exhibited a significant increase in NADPH oxidase 4 (NOX4) mRNA expression and reactive

oxygen species (ROS) production. In addition, the increased production of ROS in HSCs was associated with a significant reduction in HSC quiescence and an increase in DNA damage. These findings indicate that exposure to proton radiation can lead to long-term HSC injury, probably in part by radiation-induced oxidative stress. © 2015 by Radiation Research Society

INTRODUCTION

Since its initiation in the 1950s, human spaceflight activity has continued to increase. Unfortunately, exposure of astronauts to space radiation remains a significant health risk. During space missions, two major radiation sources are encountered: solar particle events (SPE) generated by the sun, and galactic cosmic rays (GCR) emanating from sources outside our solar system. In particular, proton radiation occupies over 80% of SPE (1–3). Astronauts may receive cumulative doses from 1–3 Gy during an SPE (4, 5). In addition, compared to traditional tumor radiation therapy, which uses X rays (photons) or electrons, proton therapy may provide an additional advantage for tumor treatment because of its favorable radiation dose distribution that can selectively increase the radiation dose to cancerous tissues while lowering dose to normal tissues (6, 7). For these reasons, there is an urgent need to better understand the biological effects of exposure to proton radiation.

It is well known that exposure to a significant dose of total-body gamma radiation (TBI) induces not only acute radiation hematopoietic syndrome but also long-term bone marrow (BM) injury (8, 9). This acute radiation hematopoietic syndrome is primarily attributed to apoptosis induction of HPCs, and the BM injury is mainly ascribed to persistent HSC damage. While the effects of gamma irradiation on the hematopoietic system have been extensively documented, much less is known about the effects of proton irradiation (10, 11). Several recent studies have reported proton radiation-induced acute effects on the

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mouse hematopoietic system (12–17). Decreased white blood cells (WBCs), lymphocytes and neutrophils were detected starting at 4 h after proton irradiation with 0.25–3 Gy, and the lowest of these levels were observed on day 4 (15, 16). By day 21 postirradiation with 2 Gy of protons (230 MeV), the reduction in WBCs and lymphocytes was still evident in mice (13). According to the data obtained 24 h post exposure, the 50% effective dose of proton radiation is 1.0 Gy (30–74 MeV) for WBCs and lymphocytes (17). It was shown that the relative proton radiation sensitivity of lymphocyte subsets was B cells > T cells > natural killer (NK) cells > T cytotoxic cells (Tc) > T helper cells (Th), which is in agreement with the results from gamma irradiation (18). These previous studies have shown almost complete recovery of the peripheral blood cell counts after proton irradiation. However, significant abnormalities in WBCs and lymphocytes in spleen at 110 days after exposure were reported (19), suggesting that proton exposure may have long-term detrimental effects on the hematopoietic system.

Therefore, in the current study we investigated the long-term effects of proton exposure on the hematopoietic system in mice. Specifically, C57BL/6J mice that were exposed to 1.0 Gy proton TBI and the long-term effects of proton irradiation on BM 22 weeks after the exposure were analyzed. Our results show that proton-radiation exposure can induce long-term BM damage in mice.

MATERIALS AND METHODS

Animals and Irradiation

Six-month-old male C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME) were shipped to Brookhaven National Laboratories (BNL, Upton, NY). After a one-week acclimation period, the mice were either sham irradiated or whole-body irradiated with 1.0 Gy of 150 MeV/n protons. One week after irradiation, the mice were shipped to Oregon Health and Science University (OHSU). At BNL and OHSU, the mice were housed under a constant 12:12 h light-dark schedule. Food (PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International, St. Louis, MO) and water were provided *ad libitum*. Behaviorally naïve mice were used for experiments. All procedures were approved by the Institutional Animal Care and Use Committee at OHSU and BNL.

Isolation of BM Mononuclear Cells and Analysis of the Frequencies and Numbers of Different Hematopoietic Cell Populations by Flow Cytometry

The femora and tibiae were harvested from mice immediately after they were sacrificed by cervical dislocation. Bone marrow cells were flushed from the bones into Hanks' balanced salt solution (HBSS) containing 2% fetal calf serum (FCS) using a 21-gauge needle and syringe, and were pre-incubated with biotin-conjugated anti-CD3e, anti-CD45R/B220, anti-Gr-1, anti-CD11b and anti-Ter-119 antibodies, as well as with anti-CD16/32 (Fcγ II/III receptor or FcγR) antibody to block the Fcγ receptors before they were labeled with streptavidin-FITC, anti-Sca-1-PE-Cy7, anti-c-Kit-APC-Cy7 for HPCs (Lin[−]Sca1⁺c-kit⁺ cells) and HSCs (Lin[−]Sca1⁺c-kit⁺ cells). Bone marrow mononuclear cells (BM-MNCs) were isolated by Histopaque® 1083 separation solution (Sigma-Aldrich® LLC, St. Louis, MO). For the isolation of lineage-negative cells (Lin[−] cells), BM-MNCs were

incubated with purified rat antibodies specific for murine CD3e, Mac-1, CD45R/B220, Ter-119, and Gr-1. The labeled mature lymphoid and myeloid cells were depleted twice by incubating with goat anti-rat IgG paramagnetic beads (Life Technologies, Grand Island, NY) at a bead:cell ratio of approximately 4:1. Cells binding the paramagnetic beads were removed with a magnetic field. Lin[−] cells were washed twice with 2% FBS/HBSS and resuspended in complete medium (RPMI1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 μM HEPES buffer and 100 U/mL penicillin and streptomycin) at 1×10^7 cells/mL. Subsequently, cells were blocked by Fcγ receptor anti-CD16/32 antibody then stained with anti-Sca1-PE-Cy7, c-Kit-APC-Cy7. All flow antibodies were purchased from eBioscience, Inc. (San Diego, CA). The frequencies of HPCs and HSCs were analyzed with a BD FACSAria™ II cell sorter (BD Biosciences, San Jose, CA). Dead cells were excluded by gating out the cells stained positive with propidium iodide. For each sample, approximately 8×10^5 to 1×10^6 BM cells were acquired and the data were analyzed using BD FACSDiva™ 6.0 (BD Biosciences) and FlowJo (FlowJo, Ashland, OR) software.

Colony-Forming Cell Assay and Cobblestone Area-Forming Cell Assay

Colony-forming cell (CFC) assay was performed by culturing BM-MNCs in MethoCult™ GF M3434 methylcellulose medium (Stem Cell™ Technologies Inc, Vancouver, Canada). Colonies of CFU-granulocyte macrophage (GM) and burst-forming unit-erythroid (BFU-E) were scored on day 7, and colonies of CFU-granulocyte, -erythrocyte, -monocyte and -megakaryocyte (GEMM) were scored on day 12 of the incubation according to the manufacturer's protocol. Cobblestone area-forming cell (CAFC) assay was performed as described in previously published articles (20, 21).

Analysis of the Levels of Intracellular Reactive Oxygen Species

To measure radiation-induced reactive oxygen species (ROS), such as hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2) and superoxide radical ($\text{O}_2^{\cdot-}$), we took advantage of the extensively used probe DCFDA to assess ROS production in HSCs after proton exposure (20, 22). Briefly, after staining with the appropriate cell surface marker antibodies, Lin[−] cells (1×10^7 /mL) were suspended in phosphate buffered saline (PBS) supplemented with 5 mM glucose, 1 mM CaCl_2 , 0.5 mM MgSO_4 and 5 mg/ml bovine serum albumin (BSA) and then incubated with 10 μM 2',7'-dichlorofluorescein diacetate (DCFDA) (Life Technologies) for 30 min at 37°C. The ROS levels in HPCs and HSCs were analyzed by measuring the mean fluorescence intensity (MFI) of 2',7'-dichlorofluorescein (DCF) with the FACSAria II cell sorter. For each sample, a minimum of 200,000 lineage negative cells was acquired and the data were analyzed as we previously described (23).

DNA Damage Analysis

Lin[−] cells were first stained with antibodies against various cell-surface markers and fixed and permeabilized using Fixation/Permeabilization Solution (BD Biosciences) followed by 0.2% Triton-X-100 incubation for 10 min. Cells were then stained with Alexa Fluor® 488 conjugated anti-phospho-histone 2AX (Ser139) antibody for 1.5 h at 4°C and analyzed by flow cytometry. The levels of DNA damage were expressed by the mean fluorescence intensity of phospho-histone 2AX or γ-H2AX with the FACSAria II cell sorter.

Cell Cycle Analysis

Lin[−] cells were first stained with antibodies against various cell-surface markers and fixed and permeabilized using Cytofix/Cytoperm™ fixation/permeabilization solution (BD Biosciences). The cells were subsequently stained with anti-Ki67-FITC antibody (BD

FIG. 1. Proton TBI causes sustained quantitative reduction of HSCs but not HPCs. C57BL/6J mice were exposed to 1.0 Gy proton TBI or sham irradiated (CTL). Twenty-two weeks after TBI, BM cells (BMCs) were harvested from the two hind legs of individual mice for analysis. Panel A: Representative gating strategy of flow cytometric analysis for HPCs (Lin⁻Sca1⁻c-kit⁺ cells) and HSCs (Lin⁻Sca1⁺c-kit⁺ cells) in BMCs is shown. Frequencies (panel B) and total numbers (panel C) of HPCs and HSCs in BMCs from each mouse are presented as mean $\pm$ SD (CTL: n = 4 mice; TBI: n = 5). * P < 0.05 and ** P < 0.01, TBI vs. CTL.

Biosciences) and 7-AAD (Sigma-Aldrich), then analyzed by flow cytometer.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN®, Valencia, CA) according to the manufacturer's instructions. RNA yield and quality were determined by measuring absorbencies at 260 and 280 nm, respectively. First-strand cDNA was synthesized in a final volume of 20 μ l using the SuperScript® III First-Strand Synthesis System (Invitrogen™, Carlsbad, CA). qRT-PCR analyses were performed using a SYBR® Green mix on the AB StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Carlsbad, CA). Hypoxanthine phosphoribosyltransferase (HPRT) transcripts were used as a housekeeping internal reference for mRNA. The expression of NOX1, NOX2, NOX4, p21, p57, cyclin D1 and cyclin D3 was calculated by the comparative C_T method as described previously (23). The sequences for all the primers used in the qRT-PCR assays are available upon request.

Statistical Analysis

All data are presented as mean $\pm$ standard error of mean (s) of at least four independent biological samples per radiation dose. The differences between sham-irradiated and irradiated groups were examined by unpaired Student's t test. Differences were considered

significant at P < 0.05. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., LaJolla, CA).

RESULTS

Proton-Radiation-Induced Long-Term HSC Injury

In a previously published study, it was demonstrated that 4 months postirradiation, proton-radiation exposure had induced an abnormal distribution of WBCs and lymphocytes in spleen even though peripheral blood cell counts were back to normal (19). Mature blood cells and their progenitors are derived from the rare HSC population in the bone marrow. It is unclear whether the abnormalities were induced by proton irradiation due to a HSC defect. To test this possibility, we exposed C57BL/6J mice to 1.0 Gy proton TBI. Twenty-two weeks after TBI when various peripheral blood cell counts and BM cell numbers in the irradiated mice had returned to normal levels (data not shown), we harvested BM from the irradiated and sham-irradiated mice and analyzed the frequencies and numbers of different hematopoietic cell populations in BM cells by flow cytometry (see Fig. 1A). The results from this assay

FIG. 2. Proton TBI causes sustained reduction of HSC clonogenic function. Panel A: Twenty-two weeks after 1.0 Gy TBI, total BM cells (BMCs) were harvested from control (CTL) and irradiated (TBI) mice and were analyzed by CAFC assays. The numbers of five week CAFCs were counted and expressed as mean $\pm$ SD ($n = 3$ –4 mice per group) of CAFCs per 100,000 BMCs. * $P < 0.05$ TBI vs. CTL. Panels B–D: At 22 weeks after TBI, BM-MNCs were isolated from irradiated and sham-irradiated mice. A CFC assay was performed as described in Materials and Methods. The results are presented as mean CFUs per 2×10^4 BM-MNCs ($n = 3$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, TBI vs. CTL.

revealed that the frequencies and numbers of HPCs (Lin[−]Sca1[−]c-kit⁺ cells) in the irradiated mice had nearly returned to normal levels (Fig. 1B and C), but the frequencies of HSCs (Lin[−]Sca1⁺c-kit⁺ cells) in these mice remained significant lower than those in sham-irradiated controls (Fig. 1B; $P < 0.01$). The negative effects of proton irradiation on the numbers of HSCs were the same as that of the frequencies of HSCs (Fig. 1C; $P < 0.05$).

Proton irradiation not only decreases HSC numbers in BM but also results in long-term alterations in HSC functions. When measured by 5 week CAFC assay, a widely used *in vitro* surrogate assay for HSCs (24), the frequencies of functional HSCs were significantly lower in BM cells from the irradiated mice than in those from normal controls (Fig. 2A; $P < 0.05$). In addition, the frequencies of BFU-Es, CFU-GMs and CFU-GEMMs were also significantly reduced in irradiated mice compared to control mice (Fig. 2B–D; $P < 0.05$, $P < 0.001$ and $P < 0.01$, respectively), indicating that the ability of HSCs to differentiate into granulocytes, erythrocytes, monocytes and/or megakaryocytes was dramatically decreased in irradiated mice compared to sham-irradiated mice (Fig. 2B–D; $P < 0.05$). These findings clearly demonstrate that proton irradiation causes long-term hematopoietic damage to HSCs.

Proton-Radiation Induced Persistent Oxidative Stress Selectively in HSCs

In our previously published studies, we showed that gamma irradiation induces long-term HSC damage primarily via induction of increased ROS production and chronic oxidative stress (21, 23, 25). Increase in ROS can stimulate HSC cycling and/or cause oxidative DNA damage, resulting in HSC exhaustion and/or induction of HSC senescence. To elucidate the role of ROS in the effects of proton irradiation on HSCs, intracellular production of ROS in different populations of hematopoietic cells in BM from proton-irradiated mice was compared with that of controls. As shown in Fig. 3A and B, ROS production in HPCs from irradiated mice was slightly higher (about 1.2-fold) than in cells from sham-irradiated mice, although this did not reach statistical significance. However, after proton TBI, ROS levels in irradiated HSCs were greater than those in HSCs from sham-irradiated control mice. This finding confirmed that proton TBI can also selectively induce chronic oxidative stress in HSCs, as total-body gamma irradiation had done in our previous studies (21, 23, 25). It is well known that ROS could be derived from mitochondria and NADPH oxidases (NOXs) in mammalian cells. NOX1, NOX2 and NOX4, but not NOX3 and NOX5, are expressed

FIG. 3. Proton TBI causes persistent increases in ROS production in HSCs but not in HPCs. Lin⁺ cells were isolated from control (CTL) and irradiated (TBI) mice 22 weeks after 1.0 Gy proton TBI as described. Panel A: Representative analysis of ROS production measured by flow cytometry using DCFDA in BM HPCs and HSCs from control and irradiated mice. The numbers presented in the histograms are DCF MFI from a representative experiment. Panel B: The levels of MFI in BM HPCs and HSCs after TBI are presented as mean $\pm$ SD (CTL: n = 4; TBI: n = 5). Panel C: The expression of NOX1, NOX2 and NOX4 mRNA in HSCs from irradiated and CTL mice was measured by qRT-PCR and expressed as fold increases compared to CTL. Data are presented as mean $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 and *** P < 0.001, TBI vs. CTL.

in mouse HSCs (25). Using the gamma-irradiation mouse model, we have shown that the gamma-radiation-induced increase in ROS production in HSCs was mainly due to the upregulation of NOX4 (25). Thus, we measured expression of NOX1, NOX2 and NOX4 in HSCs from proton-irradiated and unirradiated mice. The result showed that the level of NOX4 mRNA expression in proton-irradiated HSCs was 36-fold higher than in unirradiated HSCs, while the levels of NOX1 and NOX2 mRNA expression in irradiated HSCs were modestly increased by 1.8- and 3.0-fold, respectively (Fig. 3C).

Moreover, we utilized Ki-67 and 7-AAD double staining to investigate the cell cycle status of HPCs and HSCs. The increased production of ROS in irradiated HSCs was associated with a significant reduction in HSC quiescence (fewer G₀-phase cells, P < 0.05 and more G₁-phase cells, P < 0.01), indicating that proton-radiation-induced ROS stimulates HSC cycling and proliferation (Fig. 4A and B). We further sorted out HSCs from irradiated and sham-irradiated mice and performed gene expression analysis in HSCs. Compared to the sham-irradiated HSCs, a greater than twofold upregulation of cyclin D1 (P < 0.001) and D3 (P < 0.001) in irradiated HSCs was detected (Fig. 4C). Expression of p21, but not of p57, was downregulated in irradiated HSCs (Fig. 4C; P < 0.01). However, HPCs from proton-irradiated mice were relatively normal in all of these assays, indicating that proton irradiation can selectively affect HSCs. Increased cycling of HSCs after proton irradiation may compensate for the decrease of HSCs

induced by proton irradiation but at the expense of HSC self-renewal, which can result in long-term damage to the hematopoietic system.

Proton-Radiation Induced Sustained DNA Damage in HSCs

Since proton TBI can induce persistent oxidative stress selectively in HSCs, then impaired HSC function may be due to sustained DNA damage induced by this persistent oxidative stress (26). To test this hypothesis, we used flow cytometry to analyze MFI of γ -H2AX immunostaining in HPCs and HSCs to measure DNA damage or DNA double-strand breaks. As shown in Fig. 5A and B, HPCs and HSCs from sham-irradiated mice had low levels of γ -H2AX MFI. HPCs from irradiated mice showed no significant change in MFI of γ -H2AX staining compared to the HPCs from sham-irradiated mice. However, exposure to proton TBI resulted in a significant increase in γ -H2AX MFI in HSCs (P < 0.05). Therefore, these data demonstrate that proton irradiation induces sustained DNA damage in irradiated HSCs, which may contribute to proton-radiation-induced long-term damage to HSCs.

DISCUSSION

We demonstrate here that proton irradiation causes long-term hematopoietic injury, and that mice exposed to a 1 Gy dose of proton radiation resulted not only in a sustained reduction in frequency of BM HSCs but also in long-term

FIG. 4. Proton TBI causes cell cycling in HSCs but not in HPCs. BMCs were harvested from control (CTL) and irradiated (TBI) mice 22 weeks after 1.0 Gy proton TBI as described. Panels A and B: Percentages of G₀, G₁ and SG₂M cells in HPCs and HSCs from control and irradiated mice are presented as mean $\pm$ SD (CTL: n = 4 ; TBI: n = 5). * P < 0.05 and ** P < 0.01, TBI vs. CTL. Panel C: Fold changes in relative gene expression are shown for various cell cycle regulators in sorted HSCs after TBI. Data from three independent experiments using sorted HSCs pooled from four mice per group are presented as mean $\pm$ SD (n = 3). * P < 0.05 and *** P < 0.001, TBI vs. CTL.

inhibition of HSC clonogenic function. In contrast to those results, we found that the number and frequency of HPCs returned to a normal level at 22 weeks postirradiation. An important question that needs to be addressed is whether damage to HSC function by proton irradiation can also lead to hypoplastic syndrome after further hematopoietic stress. This is particularly important because it has been reported that myeloid leukemia can be induced by low and/or moderate doses of gamma radiation (27, 28). A previous study has shown that proton irradiation could induce hepatocellular adenoma and malignant lymphoma with rare myeloid leukemia in CBA mice (29). This may be due to the differences of relative biological effectiveness (RBE) between proton and photon radiation. Most recently, Sanzari *et al.* utilized a simulated electron solar particle event (eSPE) as the reference radiation to estimate the RBE of simulated proton solar particle event (pSPE) in the minipig model (30). They showed that pSPE radiation had significant adverse effects on the numbers of peripheral WBCs, lymphocytes, neutrophils, monocytes and eosinophils compared to eSPE radiation with the average RBE of 2.79 for pSPE. Low energy protons have a higher linear energy transfer and are a much denser ionizing radiation than photon and explains why at the end of their range, (known as the “Bragg peak”), these low energy protons have the potential to cause serious damage to target tissues, cells and molecules. Compared to gamma radiation, proton

radiation can induce larger γ -H2AX foci (31), increased hypermethylated DNA (32), different transcriptome profiles (33) and different signaling pathways (34). More detailed investigation into the unique biological effects of proton radiation is warranted to further guide proton studies.

Our findings demonstrate that proton-radiation induces long-term BM injury by inducing persistent ROS production selectively in HSCs. This finding is in agreement with our previous published studies using gamma irradiation, suggesting that both proton and gamma irradiation may induce late effects on BM, probably by induction of chronic oxidative stress in HSCs (21, 23, 25). It remains to be determined whether these chronic oxidative stressed HSCs induced by proton radiation will undergo senescence or genomic instability, and if so, whether the HSC genomic instability could eventually lead to development of leukemia, as those induced by gamma irradiation (20, 23, 35).

Using different tumor cell lines, such as PC3, MCF7, HepG2 and neural precursor cells, investigators have shown copious production of ROS after proton irradiation (34, 36, 37). Moreover, the increase in ROS production induced by proton irradiation is more rapid and results in higher levels than is found with photon irradiation in neural precursor cells. The deleterious effects of proton-radiation-induced ROS production are also indirectly supported by other studies (38), which showed that antioxidant supplements

FIG. 5. Proton TBI causes persistent DNA damage in HSCs but not that in HPCs. Lin⁺ cells were isolated from control (CTL) and irradiated (TBI) mice 22 weeks after 1.0 Gy proton TBI as described. Panel A: Representative analysis of DNA damage measured by flow cytometry using γ -H2AX staining in BM HPCs and HSCs from control and irradiated mice. The numbers presented in the histograms are γ -H2AX MFI from a representative experiment. Panel B: The levels of MFI in BM HPCs and HSCs after TBI are presented as mean $\pm$ SD (CTL: n = 4; TBI: n = 5). * P < 0.05, TBI vs. CTL.

significantly increased 30 day survival of mice when given after a high dose of proton radiation. Pretreatment of mice with antioxidants also increased total peripheral blood white cells, polymorphonuclear cells and lymphocytes after proton TBI.

It is well known that ROS can be mainly derived from mitochondria in mammalian cells. ROS are produced in mitochondria through oxidative phosphorylation during O₂ metabolism. However, HSCs reside in the unique hypoxic niche with low levels of O₂ and harbor immature mitochondria and low mitochondria mass. A significant high level of the hypoxia marker pimonidazole can also be detected in HSCs. In response to hypoxia, HSCs express higher levels of hypoxia inducible factor 1 α (HIF-1 α) compared to HPCs (39). High levels of HIF-1 α allow HSCs to use anaerobic glycolysis other than mitochondrial oxidative phosphorylation to produce energy and reduce production of ROS. Our recent studies have revealed that gamma-radiation-induced ROS production in HSCs is

unlikely derived from mitochondria, but generated by NADPH oxidases (NOXs) (25). Specifically, we found that NOX4 expression, but not other isoforms of NOXs, was upregulated in HSCs from gamma-irradiated mice (25). Inhibition of NOX4 activity by diphenyliodonium (a selective NOX inhibitor) can ameliorate radiation-induced ROS production in HSCs (25). In the current study, we demonstrated that the level of NOX4 mRNA expression in irradiated HSCs was significantly higher than that in unirradiated HSCs, suggesting that NOX4 may play a role in the induction of ROS production in HSCs after proton exposure. This hypothesis will be tested in our future studies by determining whether inhibition of NOX4 activity with a specific inhibitor can reduce TBI proton induced increase in ROS production and improve the function of HSCs after proton irradiation.

Compared to HPCs, two important features of adult HSCs are their quiescence and slow cycling (40). It has been suggested that dormant HSCs that divide only once every

145 days have the highest self-renewal potential of all blood cell populations. The HSC quiescence is important for HSC maintenance because it prevents HSC from premature exhaustion. It has been shown that genetic deletion of FOXO3a, Bmi-1 and TSC1 in mice increases ROS production and HSC cycling, which eventually causes the loss of stem cell self-renewal ability and the exhaustion of HSCs (22, 41, 42). Our results showed that there were many fewer numbers of HSCs in G₀ phase and higher numbers of HSCs in G₁ phase after proton irradiation compared to unirradiated control. Consistent with this observation we found an upregulation of cell cycle positive regulators cyclin D1 and cyclin D3 in proton-irradiated HSCs. The increased proliferation of HSCs may initially compensate for the decreased number of HSCs induced by proton irradiation but likely at the expense of HSC self-renewal. In addition, we demonstrated that proton-radiation-induced ROS production was associated with persistent increases in DNA damage in HSCs but not in HPCs. The persistent DNA damage can also contribute to proton-radiation-induced long-term damage to HSCs. Therefore, our findings provide strong evidence demonstrating that proton TBI induces long-term BM suppression, at least in part by induction of oxidative stress in HSCs. Proton-radiation-induced long-term BM injury may be treatable with a potent antioxidant, which will be tested in the future.

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