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Whole body proton irradiation causes acute damage to bone marrow hematopoietic progenitor and stem cells in mice

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Abstract

Purpose: Exposure to proton irradiation during missions in deep space can lead to bone marrow injury. The acute effects of proton irradiation on hematopoietic stem and progenitor cells remain undefined and thus were investigated.

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Jianhui Chang, his research focuses on radiation-induced senescence of hematopoietic stem cells and muscle stem cells using mice models. He is investigating how to deplete these senescent stem cells induced by irradiation.

Yingying Wang, her research focuses on the molecular mechanisms which might cause cellular senescence, contributing to investigate different strategies to deplete senescent cells in vitro and in vivo.

Rupak Pathak, his current research interest is radiation-induced normal tissue injury particularly radiation-induced endothelial dysfunction and genomic instability using FISH-based molecular techniques. One of the major research goals is to develop radiation countermeasures that can be used in the event of accidental or incidental radiation mass casualty.

Vijayalakshmi Sridharan, her research focuses on the interactions between ionizing radiation and normal tissues, particularly the molecular mechanisms that lead to the development of radiation-induced heart disease and the identification of potential treatments.

Tamako Jones, her research interest is the effects of charged particle radiations on the hematopoietic system using irradiated mice models.

Xiao Wen Mao, her research interest is to evaluate an antioxidant compound to protect the normal tissue, including normal vasculature, from radiation-induced damage. This agent can be considered for translational studies to use in radiation therapy as a therapeutic radio-protector.

Gregory Nelson, his current research interests center on the effects of ionizing radiation on normal cells and tissues. He is investigating functional consequences of radiation exposure with emphasis on charged particle radiations such as protons. His interests in astronaut health extend to effects of weightlessness and have involved experiments flown on five space shuttle missions.

Marjan Boerma, her research focuses on cancer survivorship issues, particularly radiation-induced heart disease, risks of cardiovascular disease from exposure to radiation during space travel, and development of medical countermeasures against nuclear accidents or terrorism.

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Lijian Shao, his research focuses on radiation-induced long term hematopoietic stem cell injury and on understanding the molecular mechanisms of residual stem cell damage after exposure to radiation. His goal is to development of medical countermeasures against radiation-induced hematopoietic injury in cancer patients.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Materials and methods: We exposed male C57BL/6 mice to 0.5 and 1.0 Gy proton total body irradiation (proton-TBI, 150 MeV) and examined changes in peripheral blood cells and bone marrow (BM) progenitors and LSK cells 2 weeks after exposure.

Results: 1.0 Gy proton-TBI significantly reduced the numbers of peripheral blood cells compared to 0.5 Gy proton-TBI and unirradiated animals, while the numbers of peripheral blood cell counts were comparable between 0.5 Gy proton-TBI and unirradiated mice. The frequencies and numbers of LSK cells and CMPs in BM of 0.5 and 1.0 Gy irradiated mice were decreased in comparison to those of normal controls. LSK cells and CMPs and their progeny exhibited a radiation-induced impairment in clonogenic function. Exposure to 1.0 Gy increased cellular apoptosis but not the production of reactive oxygen species (ROS) in CMPs two weeks after irradiation. LSK cells from irradiated mice exhibited an increase in ROS production and apoptosis.

Conclusion: Exposure to proton-TBI can induce acute damage to BM progenitors and LSK cells.

Keywords

Proton irradiation; hematopoietic stem cells; apoptosis; reactive oxygen species

Introduction

During spaceflight missions beyond low Earth orbit, astronauts will be exposed to space radiation. Solar particle events (SPE) are relatively unpredictable and can lead to high dose and moderate-dose rate exposures to ionizing radiation, mainly in the form of protons (Townsend 2005; Cengel et al. 2010). Because the dose-depth distribution of SPE spectrum protons is relatively low (Nelson 2016), during an SPE the skin and organs near the surface of the skin typically will receive higher doses compared to deeper organs such as the bone marrow (BM). However, the hematopoietic system is highly sensitive to ionizing radiation, and exposure to even a relatively low dose of SPE may still be able to cause substantial damage to the system (Harrington et al. 1997; Chambers et al. 1998). Due to the large difference in size between mice and human subjects, the dose-depth distribution in mice exposed to protons is different from that in humans (Cengel et al. 2010). The dose to the blood-forming tissues in mice is likely very similar to the dose at the surface, while the dose to the blood forming tissues in humans will be lower. This difference in dose-depth profile should be taken into considerations when translating risk of hematopoietic injury in response to proton exposure in a mouse model to the human situation.

One of the characteristics of the radiation-induced hematopoietic syndrome is a decline in blood cell counts, resulting from radiation-induced cell killing in circulating blood cells and suppression of hematopoietic stem and progenitor cells (Wang et al. 2006; Shao et al. 2010; Yu et al. 2010). Several recent studies have reported that whole-body exposure to protons induces acute effects on the hematopoietic system in animal models. For instance, proton (250 MeV) irradiation at doses of 0.25–3 Gy in a mouse model significantly decreased peripheral white blood cell (WBC), lymphocyte, and neutrophil counts starting from 4 h after irradiation, with the lowest cell numbers observed on day 4 (Luo-Owen et al. 2012). The threshold dose of protons (50 or 70 MeV) to induce a decline in WBC counts in a

mouse model was estimated to be between 0.25 and 0.5 Gy (Maks et al. 2011). By day 21 post-exposure to 2.0 Gy of protons (230 MeV), the reduction in WBCs and lymphocytes was still evident in mice (Gridley et al. 2008; Sanzari et al. 2013). We have previously shown that a single dose of 1.0 Gy of proton reduced the numbers and function of hematopoietic stem and progenitor cells (HSPCs) 22 weeks post exposure (Chang et al. 2015). These long-term negative effects seemed mainly mediated by chronic oxidative stress, leading to DNA damage and altered cell cycling in irradiated HSPCs.

While previous studies have shown that protons can cause decline in peripheral blood cell counts in mice at relatively low doses, there is little information in the literature about the acute effects of proton irradiation on BM HSPCs that are responsible for generation of various blood cells. Therefore, in the present study we exposed mice to two different doses (0.5 and 1.0 Gy) of proton irradiation and examined the acute effects of proton irradiation on BM HSPCs.

Materials and methods

Animals and irradiation

Male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) and housed at the University of Arkansas for Medical Sciences (UAMS) until 6 months of age. Mice were administered a standard soy-protein free rodent diet (2020X, Harlan Teklad, Indianapolis, IN, U.S.A.) and water *ad libitum* and housed under a constant 12 h light:dark cycle throughout the study. Six-month old mice were shipped to Brookhaven National Laboratories (BNL) in Upton, NY, U.S.A. After a 1-week acclimation period, the mice were either sham irradiated or received acute doses of whole-body proton irradiation (150 MeV/n; 0.5 and 1.0 Gy, 35–55 cGy/min, $n = 5$) at the NASA Space Radiation Laboratory (NSRL). Dosimetry was performed by the NSRL physics group to ensure the quality of exposure. For each exposure, animals were individually placed into clear polystyrene cubes ($3 \times 1\frac{1}{2} \times 1\frac{1}{2}$ in) with breathing holes. Sham-irradiated mice were placed into the same enclosures for the same amount of time, but were not exposed to radiation. One day after (sham-) irradiation, the mice were returned to UAMS and administered 2020X chow containing 150 ppm fenbendazole as a routine quarantine procedure, throughout the remainder of this study. During the entire experiment, sham-irradiated mice were not housed together with irradiated mice. All procedures in this study were approved by the Institutional Animal Care and Use Committees of UAMS and BNL. Because the dose-depth distribution in mice is different from that in human, the doses used in the present study to the blood-forming tissues of human should be less.

Peripheral blood and BM collection

Mice were anesthetized with 3% isoflurane inhalation at 2 weeks after radiation exposure. A modified infusion set (27G with shortened tubing) was used to inject a single dose of heparin (30–40 U/kg) into the abdominal vena cava. Without changing the position of the infusion set, a blood sample was drawn and collected into an EDTA coated tube, and stored on ice until further analysis. Peripheral blood cell numbers were determined with a Vet ABC™ Hematological analyzer (SCIL Animal Care Co.). The femora and tibia were

harvested and immediately flushed with Hanks' Balanced Salt Solution (HBSS) containing 2% fetal bovine serum (FBS) using a 21-gauge needle and syringe to collect BM. BM samples were placed on ice and analyzed within the same day as described below.

Isolation of BM mononuclear cells (BM-MNCs), analysis of the frequencies and numbers of different hematopoietic cell populations by flow cytometry

BM cells were pre-incubated with biotin-conjugated anti-CD3e, anti-CD45R/B220, anti-Gr-1, anti-CD11b, and anti-Ter-119 antibodies. Cells were then incubated with anti-CD16/32 (Fc γ II/III Receptor or Fc γ R) antibody to block the Fc γ receptors. They were labeled with streptavidin-FITC, anti-Sca-1-PECy7, anti-c-Kit-APC-Cy7 for CMPs (Lin⁻Sca1⁻c-kit⁺ cells) and LSK cells (Lin⁻Sca1⁺c-kit⁺ cells). BM-MNCs were isolated by Histopaque 1083 separation solution (Sigma, St. Louis, MO, U.S.A.). 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 by incubating with goat anti-rat IgG paramagnetic beads (Life Technologies, Grand Island, NY, U.S.A.) 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 re-suspended in complete medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 μ M HEPES buffer, and 100 U/mL penicillin and streptomycin) at 1×10^7 cells/ml. Subsequently, isolated Lin⁻ cells were blocked by Fc γ receptors anti-CD16/32 antibody and then stained with anti-Sca1-PE-Cy7 and c-Kit-APC-Cy7 without further lineage surface marker staining. All flow antibodies were purchased from eBioscience (San Jose, CA, U.S.A.). The frequencies of CMPs and LSK cells were analyzed with an Aria II cell sorter. Dead cells were excluded by gating out the cells stained positive with propidium iodide (PI). 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, U.S.A.) software.

Colony-forming cell (CFC) assay

The CFC assay was performed by culturing BM-MNCs in MethoCult GFM3434 methylcellulose medium (Stem Cell Technologies, Vancouver, BC, 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) on day 12 of the incubation according to the manufacturer's protocol.

Analysis of the levels of intracellular reactive oxygen species (ROS)

After staining with the appropriate cell surface marker antibodies, Lin⁻ cells (1×10^7 /ml) were suspended in PBS supplemented with 5 mM glucose, 1 mM CaCl₂, 0.5 mM MgSO₄, and 5 mg/ml BSA and then incubated with 10 μ M 2',7'-dichlorofluorescein diacetate (DCFDA) (Life Technologies, Grand Island, NY, U.S.A.) for 30 min at 37 °C. The levels of ROS in CMPs and LSK cells were analyzed by measuring the mean fluorescence intensity (MFI) of 2',7'-dichlorofluorescein (DCF) with an Aria II cell sorter. For each sample, a minimum of 200,000 lineage negative cells was acquired, and the data were analyzed as previously described.

DNA damage analysis

Lin⁻ cells were first stained with antibodies against various cell-surface markers and fixed and permeabilized using the Fixation/Permeabilization Solution from BD Biosciences (San Diego, CA, U.S.A.) followed by 0.2% Triton-X-100 incubation for 10 min. Cells were then stained with Alexa Fluor 488 conjugated anti-phospho-Histone 2AX (γ H2AX) (Ser139) antibody for 1.5 h at 4 °C and analyzed by flow cytometry. The levels of DNA double-strand break damage were expressed by the mean fluorescence intensity of γ H2AX with an Aria II cell sorter.

Cell cycle analysis

Lin⁻ cells were first stained with antibodies against various cell-surface markers and fixed and permeabilized using the Fixation/Permeabilization Solution (BD Biosciences). Subsequently, they were stained with anti-Ki67-FITC antibody (BD Biosciences) and 7-aminoactinomycin (7-AAD, Sigma, St. Louis, MO, U.S.A.) and then analyzed by flow cytometry.

Statistical analysis

All data are presented as mean $\pm$ standard deviation of five independent biological samples per radiation dose. The differences between sham-irradiated and irradiated groups were examined by one-way ANOVA, followed by Tukey-Kramer tests for individual comparisons. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc. LaJolla, CA, U.S.A.).

Results

Proton irradiation decreased peripheral blood cell counts

In the present study, mice were exposed to two different doses of 150 MeV proton irradiation, and peripheral blood counts were measured at 2 weeks after the radiation exposure as shown in Figure 1. The data indicated that the numbers of peripheral WBCs, lymphocytes, monocytes and neutrophils were significantly decreased after 1.0 Gy of proton exposure compared to 0.5 Gy proton TBI and non-irradiated mice ($p < 0.05$ to $p < 0.001$). However, although there was a trend towards decreased WBC, lymphocyte, monocyte and neutrophil counts after 0.5 Gy exposure, the differences didn't reach statistically significant compared to unirradiated mice. There were decreased levels of RBCs, hemoglobin (Hb) and platelets (PLT) after 1.0 Gy compared to 0.5 Gy and non-irradiated mice ($p < 0.01$ to $p < 0.001$). The levels of RBCs, Hb and PLT after 0.5 Gy were not significantly different from normal levels.

Proton irradiation damaged BM hematopoietic stem and progenitors

As described above, all of the WBC, RBC and PLT counts were decreased upon 1.0 Gy proton TBI. These cells are derived from hematopoietic stem cell that differentiate into blood cells through different lineages of progenitors, suggesting that proton irradiation could damage either stem cells or progenitor cells, or both. In the present study, we referred Lin⁻Scal⁻c-kit⁺ cells as common myeloid progenitors (CMPs) and Lin⁻Scal⁺c-kit⁺ cells as LSK

cells. LSK cells contain both hematopoietic stem cells and multipotent progenitor cells. To investigate the effect of proton TBI on these populations, the frequencies and numbers of CMPs and LSK cells in BM were measured by flow cytometry 2 weeks after 0.5 and 1.0 Gy proton exposure, as shown in Figure 2(A). The data demonstrated that proton TBI caused a significant decrease, not only in the frequencies of CMPs and LSK cells, but also their total numbers in the BM in a dose-dependent manner (Figure 2(B,C)), $p < 0.05$ to $p < 0.01$.

To assess the function of stem cells and progenitors, an in vitro colony-forming assay was conducted as shown in Figure 3. The results demonstrated that the numbers of CFU-GMs and CFU-GEMMs were significantly reduced in all proton exposed mice compared to those in non-irradiated mice ($p < 0.05$ – 0.01). The numbers of BFU-Es under 1.0 Gy exposure were significantly decreased compared to 0.5 Gy proton TBI and non-irradiated mice, while the numbers of BFU-E from 0.5 Gy irradiated mice were comparable to those in non-irradiated controls. These data indicated that the irradiated stem cells and progenitors had a dysfunction to differentiate into granulocytes, erythrocytes, monocytes and/or megakaryocytes.

Proton irradiation caused cellular apoptosis in BM CMPs and LSK cells

It is well known that hematopoietic cells are highly sensitive to radiation-induced apoptosis (Shao et al. 2014). The externalization of phosphatidylserine is one of the characteristics of cellular apoptosis, which can be easily detected by Annexin V staining. We therefore utilized Annexin V staining to detect cellular apoptosis in CMPs and LSK cells (Figure 4(A)). A dose of 1.0 Gy proton irradiation significantly increased the percentage of apoptotic CMPs compared to 0.5 Gy and non-irradiated mice ($p < 0.05$ to $p < 0.01$), while there were comparable levels of apoptosis in CMPs after 0.5 Gy irradiation compared to non-irradiated controls. The percentages of apoptotic cells in LSK cells increased in a dose-dependent manner, and 1.0 Gy proton TBI induced a significant increase, from 4% in controls to 10% after irradiation ($p < 0.05$).

The increase in apoptosis in CMPs and LSK cells upon proton exposure might be a result of radiation-induced DNA damage. Radiation induces several types of DNA damages including base damage, cross linking, single-strand breaks and double-strand breaks (DSBs). DSBs are a detrimental DNA damage to injured cells, which can be detected by γ -H2AX staining. We thus analyzed the mean fluorescence intensity (MFI) of γ -H2AX staining in BM CMPs and LSK cells from non-irradiated and irradiated mice (Figure 4(C)). The results showed that there were comparable levels of γ -H2AX MFI in CMPs and LSK cells among all groups, indicating that DNA DSBs induced by proton irradiation might have been repaired in irradiated CMPs and LSK cells or the cells with DNA DSBs have died out via apoptosis 2 weeks after irradiation.

Proton irradiation induced oxidative stress in LSK cells

We used ROS probe DCFDA to examine the intracellular ROS production in CMPs and LSK cells from proton-irradiated mice. As shown in Figure 5(A), there was a trend for an increased ROS production in irradiated CMPs, but without reaching statistical significance, after 0.5 and 1.0 Gy proton TBI compared to that in non-irradiated controls. However, the

levels of ROS production in LSK cells from 0.5 and 1.0 Gy irradiated mice were much higher than those in cells from non-irradiated mice ($p < 0.01$ to $p < 0.001$) and the increase appeared to be dose-dependent.

The increased ROS production induced by proton TBI might also increase proliferation of stem cells and progenitors, leading to the exhaustion and reduction in CMPs and LSK cells after irradiation. To test this possibility, Ki-67 and 7-AAD double staining was used to assess the cell cycle status of CMPs and LSK cells from non-irradiated and irradiated mice. As shown in Figure 5(B), the percentages of CMPs and LSK cells in G₀ phase were comparable between non-irradiated and irradiated mice, regardless of radiation doses. These data indicate that proton irradiation induce increased production of ROS in LSK cells, but this increase does not affect cell cycle in the irradiated CMPs or LSK cells after irradiation.

Discussion

Our present study showed that exposure of mice to 1.0 Gy of proton irradiation resulted in a statistically significant decrease in the number of circulating WBCs and PLTs 2 weeks after exposure. We previously demonstrated that 1.0 Gy of oxygen ion radiation (¹⁶O TBI) significantly decreased the cell counts of peripheral blood leukocytes when measured 2 weeks after exposure in male C57BL/6 mice (Chang et al. 2016). Interestingly, the decrease of peripheral blood cell counts was not observed 2 weeks after 0.5 Gy proton TBI in the current study, or 2 weeks after 0.1 and 0.25 Gy ¹⁶O TBI in our previous experiment (Chang et al. 2016). Previously, the threshold dose of protons (50 or 70 MeV) to induce a decline in WBC counts in female ICR out-bred mice was estimated to be between 0.25 and 0.5 Gy (Maks et al. 2011). The threshold dose identified from this previous study is lower compared to the result from our study. It may have resulted in part from the use of different strains of mice, the times when the mice were studied after radiation or the difference in energies of protons between the two studies. For example, the linear energy transfer (LET) values for 50, 70, and 150 MeV protons would be 1.26, 0.96 and 0.55 keV/mm. Because of the dose-depth distribution of SPE protons, only relatively large, or higher energy spectrum SPEs may lead to BM exposure to these doses of protons in astronauts. Nonetheless, the doses of SPEs that can cause significant HSPC damage have been observed, raising the concern that astronauts might experience reductions in circulating blood cell counts and BM HSPC damage if they encounter such an SPE. The dose-depth distribution in mice exposed to protons is different from that in humans (Cengel et al. 2010). We here found that a dose of 0.5 Gy protons (150 MeV) significantly reduced hematopoietic stem/progenitor cell function. The effects of protons at this energy and at doses below 0.5 Gy are unknown. Since the dose to the blood-forming tissues of human subjects will likely be low, future studies need to examine the hematopoietic effects of proton doses below 0.5 Gy.

Our study has demonstrated that proton TBI can acutely induce the decrease of all lineages of peripheral blood cells. Since all lineages of blood cells are derived from hematopoietic stem cells through their differentiation into progenitors. In the present study, we measured the changes of LSK cells using Lin⁻Sca1⁺c-Kit⁺ surface markers after proton TBI. LSK cells are a mixed population including hematopoietic stem cells and multipotent progenitor cells. Defined HSCs should further be investigated by combining LSK cells with CD150 and

CD48 surface makers under proton irradiation condition (Doulatov et al. 2012). Currently, our data indicate that the exposure to both 0.5 and 1.0 Gy proton TBI damaged not only CMPs but also LSK cells, leading to decreases in their numbers and function, which were supported by the decreased abilities to form in vitro colonies including BFU-E, CFU-GM and CFU-GEMM. The in vivo functional defect of hematopoietic stem and progenitor cells after proton exposure will be further investigated through bone marrow transplantation in our future studies. We have previously shown that proton irradiation induced long-term BM suppression (Chang et al. 2015). Hence, CMPs and LSK cells likely do not recover to normal levels after the damage. We have also reported that a 1.0 Gy dose of ^{16}O TBI significantly decreased peripheral blood counts and BM HSPCs 2 weeks after the exposure (Chang et al. 2016). Therefore, the mechanisms of space irradiation-induced acute damage to the hematopoietic system should be investigated further. In addition, the protons in the current study were delivered within a few minutes of time, and most charged particle exposures during space flight occur at a very low dose rate and/or fractionated. Despite we acknowledge that the high dose rates used here are a limitation of the current study, low-dose rate exposures were not possible because of practical constrains. Future studies with low-dose rate or fractionated exposures should provide further insight into dose rate dependence of hematopoietic stem/progenitor cell response to high energy charged particle irradiation.

Radiation-induced cell damage might be mediated by induction of apoptosis, DNA damage, and oxidative stress (Dorr and Meineke 2011; Shao et al. 2014). We therefore assessed these parameters in CMPs and LSK cells after proton exposure. Our data indicate that CMPs and LSK cells may respond differently to proton irradiation. Exposure to 1.0 Gy protons resulted in an increase in cellular apoptosis in CMPs. Irradiated LSK cells, on the other hand, showed both increased apoptosis and oxidative stress. Neither of the two cell types showed enhanced DNA damage or cell cycling 2 weeks after proton exposure.

Intriguingly, the induction of oxidative stress was detected in LSK cells 2 weeks after proton exposure in the current study and at 22 weeks in our previous experiment (Chang et al. 2015). The long-term increase in ROS production in LSK cells has also been reported for ^{16}O irradiation and γ -rays (Li et al. 2011; Zhang et al. 2013; Shao et al. 2014). These data suggest that oxidative stress in LSK cells may be central in the BM response to ionizing radiation. ROS production is also a major mediator of hematopoietic stem cell dysfunction in several genetic mouse models, such as $\text{TSC1}^{-/-}$ mice and $\text{Bmi-1}^{-/-}$ mice (Chen et al. 2008; Schuringa and Vellenga 2010). We have previously shown that NADPH oxidase 4 (NOX4) up-regulation in irradiated stem cells may be involved in the long-term increase in ROS production (Wang et al. 2010; Chang et al. 2015). Hence, NOX4 inhibitors such as diphenyliodonium, might be good candidates to mitigate the negative effects of proton irradiation in HSPCs. Other antioxidants, such as N-acetylcysteine and gamma-tocotrienol may also reduce ROS production to improve the function of progenitors and stem cells after proton irradiation. We will investigate the protective effects of these anti-oxidants on HSPCs after space radiation exposure in our future studies.

This study exposed mice to protons of a single energy (150 MeV). However, an SPE contains protons of multiple energies below 150 MeV. Therefore, some groups have

developed cell culture or animal models of exposure to broad energy spectra of protons to better simulate an SPE. Previously, some differences were found in peripheral blood cell counts between mice exposed to one-energy protons (230 MeV, 2 Gy) and mice exposed to SPE-like protons at the same dose level (Gridley et al. 2008). Exposure of minipigs to SPE-like protons at a skin dose as low as 0.5 Gy (estimated dose to the BM: 0.42 Gy) caused declines in peripheral blood cell counts up to at least 2 weeks after exposure (Sanzari et al. 2014). Hence, further studies with SPE-like proton exposures at low doses are warranted. Since the current study involved only two radiation doses (0.5 and 1 Gy), we were not able to prepare dose-response curves for the effects observed. In one of our separate studies, mice were irradiated with fully modulated beams containing particles from 0 energy to 150 MeV and a uniform dose versus depth profile. Doses of protons were 0.1, 0.25 and 0.5 Gy. Bone marrow cells were collected at 2 weeks after irradiation and examined as described in the current manuscript. We found a dose-dependent decrease in LSK cells, together with an increase in ROS levels and apoptosis in these cells (data not shown). In summary, our present study has demonstrated that acute exposures to medium doses of proton-TBI induced damage to CMPs and LSK cells in a mouse model. Cellular apoptosis and oxidative stress might contribute to the adverse effects of proton irradiation on the hematopoietic system.

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Figure 1.

Blood cell counts were decreased 2 weeks after proton exposure. C57BL/6J mice were exposed to 0.5 and 1.0 Gy doses of proton irradiation or were sham-irradiated as a control (CTL). The cell counts in peripheral blood were determined 2 weeks after radiation exposure. The numbers of WBCs, lymphocytes, monocytes, and neutrophils were significantly reduced after a dose of 1.0 Gy only. The numbers of RBCs and the level of Hb were decreased after 1.0 Gy compared to CTL. While the platelet counts (PLT) were reduced after 1.0 Gy, the mean platelet volume (MPV) was unaltered. Data are presented as mean $\pm$ SD ($n = 5$). The statistical significance of differences among control and experimental groups is indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by one-way ANOVA, followed by Tukey-Kramer tests for individual comparisons.

Figure 2.

Proton TBI caused reductions in numbers of CMPs and LSK cells at 2 weeks after irradiation. (A) Representative gating strategy of flow cytometric analysis for CMPs (Lin⁻Sca1⁻c-kit⁻ cells) and LSK cells (Lin⁻Sca1⁺c-kit⁺ cells) from 1.0 Gy and sham-irradiated (CTL) BM samples. (B and C) Frequencies and total numbers of CMPs (panel B) and LSK cells (panel C) are presented as mean $\pm$ SD ($n = 5$). The statistical significance of differences among control and experimental groups is indicated by asterisks: * $p < 0.05$, ** $p < 0.01$ as determined by one-way ANOVA, followed by Tukey-Kramer tests for individual comparisons.

Figure 3.

Proton TBI caused reductions in HSPC clonogenic function, independent of proton doses. At 2 weeks after TBI, BM-MNCs were isolated from irradiated and sham-irradiated (CTL) mice. A CFC assay was performed, and the results were presented as mean CFUs per 1×10^5 BM-MNCs. Data are presented as mean $\pm$ SD ($n = 5$). The statistical significance of differences among control and experimental groups is indicated by asterisks: * $p < 0.05$ as determined by one-way ANOVA, followed by Tukey-Kramer tests for individual comparisons.

Figure 4.

Proton TBI caused cellular apoptosis in CMPs and LSK cells two weeks after 1.0 Gy exposure. (A and B) Cells were stained with Annexin V to determine cellular apoptosis. Representative analysis of apoptosis measured in Lin⁻ cells by flow cytometry using Annexin V staining in BM CMPs and LSK cells from control (CTL) and 1.0 Gy proton TBI mice (A). Percentages of Annexin V positive cells are presented as mean $\pm$ SD (B, $n = 5$). (C) The histograms indicate γ H2AX mean fluorescence intensity (MFI) in BM CMPs and LSK cells after proton TBI and data are presented as mean $\pm$ SD ($n = 5$). The statistical significance of differences among control and experimental groups is indicated by asterisks: * $p < 0.05$, ** $p < 0.01$ as determined by one-way ANOVA, followed by Tukey-Kramer tests for individual comparisons.

Figure 5.

Proton TBI causes ROS production in irradiated LSK cells. (A and B) Analysis of ROS production as measured by flow cytometry using DCFDA in BM CMPs and LSK cells. The histograms indicate DCF MFI in a representative experiment from control (CTL) and 1.0 Gy proton TBI mice (A). The DCF MFI in BM CMPs and LSK cells are presented as mean $\pm$ SD (B, $n=5$). (C) Lin^- cells were isolated from control and irradiated mice 2 weeks after proton TBI. Cell cycle was measured by flow cytometry using Ki-67 and 7-AAD double staining. The percentages of CMPs and LSK cells in G0 phase are presented as mean $\pm$ SD ($n=5$). The statistical significance of the differences among control and experimental groups is indicated by asterisks: ** $p < 0.01$, *** $p < 0.001$ as determined by one-way ANOVA, followed by Tukey-Kramer tests for individual comparisons.