Corrections

MEDICAL SCIENCES

The authors note that their conflict of interest statement was omitted during publication. The authors declare that John Wong has a research service contract with Gulmay Medical, Inc. in the transfer of the SARRP technology from Johns Hopkins to the company. The SARRP was used by Dr. Xu Cao to irradiate his study animals, and was only peripherally related to the subject matter of the manuscript. Additionally, the specific unit was constructed at Johns Hopkins with grant support from the National Cancer Institute (US), and not by Gulmay.

DEVELOPMENTAL BIOLOGY

The authors note that the title appeared incorrectly. The title should instead appear as “Transcription factor genes Smad4 and Gata4 cooperatively regulate cardiac valve development.” The online version has been corrected.
Irradiation induces bone injury by damaging bone marrow microenvironment for stem cells

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Edited* by Peter N. Devreotes, Johns Hopkins University School of Medicine, Baltimore, MD, and approved December 16, 2010 (received for review October 14, 2010)

Radiation therapy can result in bone injury with the development of fractures and often can lead to delayed and nonunion of bone. There is no prevention or treatment for irradiation-induced bone injury. We irradiated the distal half of the mouse left femur to study the mechanism of irradiation-induced bone injury and found that no mesenchymal stem cells (MSCs) were detected in irradiated distal femora or nonirradiated proximal femora. The MSCs in the circulation doubled at 1 week and increased fourfold after 4 wk of irradiation. The number of MSCs in the proximal femur quickly recovered, but no recovery was observed in the distal femur. The levels of free radicals were increased threefold at 1 wk and remained at this high level for 4 wk in distal femora, whereas the levels were increased at 1 wk and returned to the basal level at 4 wk in nonirradiated proximal femur. Free radicals diffuse ipsilaterally to the proximal femur through bone medullary canal. The blood vessels in the distal femora were destroyed in angiographic images, but not in the proximal femora. The osteoclasts and osteoblasts were decreased in the distal femora after irradiation, but no changes were observed in the proximal femora. The total bone volumes were not affected in proximal and distal femora. Our data indicate that irradiation produces free radicals that adversely affect the survival of MSCs in both distal and proximal femora. Irradiation injury to the vasculatures and the microenvironment affect the niches for stem cells during the recovery period.

Results

To investigate the mechanism of irradiation-induced bone injury, we irradiated mouse femora by using a unique SARRP (Fig. 1A). The irradiation can be delivered at an accuracy of 1 mm resolution with the aid of micro-CT and X-ray. Groups of mice were irradiated at the distal half of the femora with a dose of 4 Gy once per day for 5 d consecutively. The left half of the proximal femora and the right femora did not receive irradiation as controls (Fig. 1 B and C). Histologic staining of the femoral sections showed that irradiation induced adipogenesis in the irradiated left distal femur at 1 wk relative to the right nonirradiated femur. Adipocytes were significantly increased at 4 wk after radiation in the medullary cavity (Fig. 1D). The results indicate that irradiation changes the bone marrow microenvironment.

We then examined whether irradiation injured bone and bone cells directly. The total bone volumes were not affected in both the left proximal and distal femora 1 or 4 wk after irradiation in CT analysis (Fig. 2A), and the trabecular bone thickness, num-


The authors declare no conflict of interest.

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ber, and separation were not changed either (Fig. 2 B–D). Bone histomorphometry analysis showed that numbers of both osteoclasts and osteoblasts were decreased in the distal femora after irradiation, but no changes were observed in the proximal femora (Fig. 2 E and F). The results indicate that irradiation affects osteoblasts and osteoclasts in the distal femora, but has no significant effect on differentiated osteoclasts and osteoblasts in the proximal femora (i.e., nonirradiated area).

The effects of irradiation on bone marrow MSCs were examined in CFU-fibroblast (CFU-F) assay with bone marrow cells isolated from left distal femora (i.e., irradiated area) or left proximal femora (i.e., nonirradiated area). No colonies of MSCs were observed in the left proximal femur or distal femur at 1 wk after irradiation, whereas colonies of CFU-F and CFU-osteoblast (CFU-Ob) were observed in the nonirradiated right femur (Fig. 3 A and B). Interestingly, recovery of MSCs was observed in the left proximal femur at 4 wk, but not for irradiated distal femur. Similar results were observed and osteoblast differentiation potential of MSCs in CFU-Ob assay (Fig. 3 A and C). The colonies of MSCs in the left and right nonirradiated tibia were not changed (Fig. 3 D). MSCs have been shown to be mobilized in the circulation in response to tissue damage. We therefore examined the effects of radiation on the levels of MSCs in the circulation. Previously, we have demonstrated the CD29$^+$Sca-1$^+$CD11b$^-$ bone marrow stroma are subset of MSCs (19). Flow cytometry analysis of CD29$^+$Sca-1$^+$CD45$^+$CD11b$^-$ MSCs in peripheral blood collected from the mice at 1 and 4 wk after treatment in the irradiation and sham groups indicates that CD29$^+$Sca-1$^+$CD45$^+$CD11b$^-$ MSCs were doubled at 1 wk after irradiation and increased fourfold in 4 wk (Fig. 3 E). The level of CD29$^+$Sca-1$^+$CD45$^+$CD11b$^-$ MSCs was also increased in proximal femur at 4 wk after irradiation whereas the levels in the right femur and left distal femur were unchanged. The results reveal that bone marrow MSCs can be affected indirectly following irradiation at another site in the bone marrow cavity. The increased level of Sca-1–positive MSCs in response to irradiation suggests that there is a systemic response resulting in mobilization of MSCs for the repair of bone injury.

To examine the mechanism of irradiation-induced damage of bone marrow MSCs, we measured the levels of free radicals in the bone marrow isolated from the irradiated left distal femur, nonirradiated proximal left femur, and right femur by using thiobarbituric acid reactive substances (TBARS) assay. This assay measures the consequences of lipid peroxidation and malondialdehyde specifically. The levels of TBARS were increased threefold at 1 wk and remained at the same level for 4 wk in left distal femora (Fig. 4 A), whereas the levels were increased at 1 wk and decreased to the basal level at 4 wk in nonirradiated proximal femur (Fig. 4 A). No changes of TBARS were observed in the right femora (Fig. 4 A) and circulation (Fig. 4 B), indicating that the free radicals diffuse to ipsilaterally distal femur through bone medullary channel, leading to MSCs damage. The results also suggest that irradiation produces free radicals that adversely affect the MSCs in both distal and proximal femora, but have no significant effects on differentiated osteoclasts and osteoblasts in the ipsilateral proximal femur (i.e., nonirradiated area).

Vasculatures are essential for the maintenance of MSCs as their niche (20, 21). Bone formation is coupled with angiogenesis (22–24). Angiographic images of microphil-perfused femora were performed to analyze the effects of irradiation on bone marrow vasculatures. The results showed that the blood vessels
in the irradiated left distal femora were damaged at 1 wk and 4 wk after irradiation, but the vessels were intact in the non-irradiated left proximal femora (Fig. 4C). Morphometric analysis showed that vessel volume, thickness, and number were decreased in the distal femur relative to controls (Fig. 4D–F). The results suggest that direct irradiation, not free radical generation, damages the bone marrow vasculatures.

Discussion

In this study, precise irradiation of the mouse distal femur was performed with a unique SARRP built with integration of CT, X-ray, and irradiation in a single treatment platform. The mouse distal half of the femur was precisely irradiated at accuracy of 1 mm. Here, the direct and indirect effects of irradiation on bone injury and bone marrow MSCs were examined in the same mouse femur with common bone marrow cavity. Our results reveal that irradiation can affect bone marrow MSCs indirectly in the proximal femur without direct irradiation. Interestingly, no colony formation of MSCs was observed in CFU-F and CFU-Ob assays in irradiated and nonirradiated areas of the bone marrow, and the levels of free radicals were dramatically increased. Most likely, the free radicals produced changed the microenvironment in the bone marrow and likely brought the MSCs into a quiescent phase in the nonirradiated area of bone marrow. Stem cells of hematopoietic and mesenchymal origin have been shown in the recovery of ionizing irradiation-induced distal epithelial damage (15–17).

Bone injury and fractures often occur in patients receiving curative doses of ionizing radiation. These fractures often heal very slowly and sometimes fail to unite. These clinical observations are in accord with our findings in this mouse model and suggest that irradiation damages the bone marrow microenvironment or the niches for stem cells. Recovery of bone injury from ionizing irradiation may occur from repopulation of inhabitant stem cells (14) or repopulation from the systemic circulation. Our results showed that MSCs in the direct irradiated area did not recover, whereas the MSCs in the nonirradiated proximal left proximal femur recovered quickly. The free radicals produced changed the microenvironment in the bone marrow and likely brought the MSCs into a quiescent phase in the nonirradiated area of bone marrow. Stem cells of hematopoietic and mesenchymal origin have been shown in the recovery of ionizing irradiation-induced distal epithelial damage (15–17).

The bone marrow with damaged microvasculature is not likely able to retain stem cells and lead to refractory of bone injury and fractures. It has been implicated that mobilization of stem cells in the circulation can occur in response to irradiation (25–27). Indeed, the levels of Sca-1–positive MSCs were increased in the circulation after irradiation. Different markers have been used for MSCs. A recent study revealed that bone barrow MSCs are nestin-positive cells (28). It will be interesting to investigate whether the mobilized Sca-1–positive MSCs are also nestin-positive and if that contributes to the recovery of MSCs in the bone marrow of proximal femur. It will also be interesting to investigate whether the mobilized Sca-1–positive MSCs contribute to the recovery of MSCs in the bone marrow of proximal femur.

The therapeutic potential of bone marrow-derived MSCs has recently been implicated. Not only are these cells able to migrate to injured tissues, but they also are able to differentiate into different phenotypes according to the tissues in which they reside (29). Furthermore, it has been shown that MSCs of bone marrow-
origin could hasten hematopoietic recovery and provide a better tool for cell therapy if applied autologously to patients who undergo irradiation (30, 31). Thus, improving bone marrow microenvironment and accelerating vasculogenesis could be a potential treatment for patients with radiation-induced bone injury.

Methods

Mice. C57BL/6j (WT) mice were purchased from Charles River. Male mice 8 wk of age were anesthetized by inhalation of isoflurane during the procedure and immobilized using fixtures. SARRP developed by researchers at the Department of Radiation Oncology at Johns Hopkins University was used to irradiate the mice. A clinically applicable dose rate (4 Gy min⁻¹) was delivered by using the 225-kVp setting with a 3.0-mm focal spot with a maximum 13 mA beam current. Irradiation was once per day, and the total dose is 20 Gy. All animals were maintained in the animal facility of the Johns Hopkins University School of Medicine. The experimental protocol was reviewed and approved by the institutional animal care and use committee of the Johns Hopkins University.

Histochemistry and Histomorphometric Analysis. At the time of euthanasia, the bone tissues were resected and fixed in 10% buffered formalin for 48 h, decalcified in 10% EDTA (pH 7.0) for 20 d, and embedded in paraffin. Longitudinally oriented sections of bone 4μm thick, including the metaphysis and diaphysis, were processed for H&E staining. Sections were microphotographed to perform histomorphometric measurements on the highlighted areas of the bone displayed on the digitalized image. Quantitative histomorphometric analysis was conducted in a blinded fashion with OsteoMeasureXP Software (OsteoMetrics). Two-dimensional parameters of trabecular bone were measured in a 2-mm square 1 mm distal to the lowest point of the growth plate in the trabecular bone.

CFU-F and CFU-Ob Assays. At the time of euthanasia, the femora were cut in half into proximal and distal pieces and bone marrow from medullary cavities were collected individually, and cell number was determined after removal of red blood cells with Zapoglobin (Coulter). The number of CFU-Fs and CFU-ObS in murine bone marrow isolates and in cultures of bone marrow cells was determined in cocultures with irradiated guinea pig marrow cells as reported.

Briefly, marrow cells were obtained from the femurs and tibiae of 2-mo-old female Hartley guinea pigs by flushing with a 22-gauge needle and resuspended. Cells were γ-irradiated with a 57Co source for 50 min at 1.2 Gy/min as reported. After rinsing by centrifugation, cells were resuspended in minimal essential medium-α with 20% FBS, counted, and cultured at 2.5 × 10⁶ per well of a six-well plate.

For assay of CFU-F and CFU-Ob number, 1 × 10⁶ murine marrow cells were plated into six-well plates in 3 mL minimum essential medium-α supplemented with glutamine (2 mM), penicillin (100 U/mL), streptomycin sulfate (100 μg/mL), and 20% lot-selected FBS. Duplicate cultures were established. After 2 to 3 h of adhesion, unattached cells were removed, and 2.5 × 10⁶ irradiated guinea pig feeder cells (provided by Brendan J. Canning, Johns Hopkins Asthma and Allergy Center, Baltimore) were added to cultivation medium of adherent cultures just after washing. On day 14, cultures were fixed and stained with 0.5% crystal violet. The colonies containing 50 or more cells were counted. For CFU-Ob assay, the cells were cultured with osteogenic medium as described earlier for 21 d and analyzed with Alizarin red staining. The colony-forming efficiency was determined by number of colonies per 10⁵ marrow cells plated.

FACS Analysis. Cells aliquots were incubated for 20 min at 4°C with antibodies conjugated by phycoerythrin, FITC, peridinin chlorophyll protein, and allophycocyanin against mouse Sca-1, CD29, CD45, and CD11b (Biolegend). Acquisition was performed on a FACS Aria sorter (BD Biosciences), and analysis was performed using a FACS DIVE software, version 6.1.3 (BD Biosciences).

Measurement of Lipid Oxidation. Peripheral venous blood was drawn into syringes containing preservative-free heparin (25 U/mL; Gibco/Life Technologies) and centrifuged at 1,200 × g for 10 min at 5°C to 10°C to isolate the plasma. Bone marrow samples were collected into preservative-free heparinized saline solution (25 U/mL) and centrifuged at 3,500 rpm for 10 min at 5°C to 10°C to isolate bone marrow plasma. Plasma lipid oxidation was assessed by determining the level of TBARS by using a TBARS assay kit (Zeptometrix).
Micro-CT Angiography Analysis. Blood vessels in bone were imaged by angiography of microphil-perfused long bones. The thoracic cavity was opened, and the inferior vena cava was severed after animals were killed. The vasculature was flushed with 0.9% neutral buffered saline solution containing heparin sodium (100 U/mL) through a needle inserted into the left ventricle. The vasculature was injected with a radiopaque silicone rubber compound containing lead chromate (Microfil; Flow Tech). Samples were stored at 4 °C overnight for contrast agent polymerization. Mouse femurs were dissected from the specimens and soaked for 4 d in 10% neutral buffered formalin to ensure complete tissue fixation. Specimens were subsequently treated for 48 h in a formic acid-based solution (Cal-Ex II) to decalcify the bone and facilitate image thresholding of the femoral vasculature from the surrounding tissues. Images were obtained using a high resolution (9-μm isotropic voxel size) micro-CT imaging system (MicroCT40; Scanco). A threshold of 306 was initially chosen based on visual interpretation of thresholded 2D tomograms.

Statistics. Data are presented as mean ± SEM; n = 10 (*P < 0.05).