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Lowering iron level protects against bone loss in focally irradiated and contralateral femurs through distinct mechanisms

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ABSTRACT

Radiation therapy leads to increased risk of late-onset fragility and bone fracture due to the loss of bone mass. On the other hand, iron overloading causes osteoporosis by enhancing bone resorption. It has been shown that total body irradiation increases iron level, but whether the systemic bone loss is related to the changes in iron level and hepcidin regulation following bone irradiation remains unknown. To investigate the potential link between them, we first created an animal model of radiation-induced systemic bone loss by targeting the mid-shaft femur with a single 2 Gy dose of X-rays. We found that mid-shaft femur focal irradiation led to structural deterioration in the distal region of the trabecular bone with increased osteoclasts surface and expressions of bone resorption markers in both irradiated and contralateral femurs relative to non-irradiated controls. Following irradiation, reduced hepcidin activity of the liver contributed to elevated iron levels in the serum and liver. By injecting hepcidin or deferoxamine (an iron chelator) to reduce iron level, deterioration of trabecular bone microarchitecture in irradiated mice was abrogated. The ability of iron chelation to inhibit radiation-induced osteoclast differentiation was observed in vitro as well. We further showed that ionizing radiation (IR) directly stimulated osteoclast differentiation and bone resorption in bone marrow cells isolated not from contralateral femurs but from directly irradiated femurs. These results suggest that increased iron levels after focal radiation is at least one of the main reasons for systemic bone loss. Furthermore, bone loss in directly irradiated bones is not only due to the elevated iron level, but also from increased osteoclast differentiation. In contrast, the bone loss in the contralateral femurs is mainly due to the elevated iron level induced by IR alone. These novel findings provide proof-of-principle evidence for the use of iron chelation or hepcidin as therapeutic treatments for IR-induced osteoporosis.

Abbreviations: BMD, bone mineral density; BV/TV, bone volume fraction; BS, bone surface; BFR/BS, bone formation rate/bone surface; CFU-F, CFU-fibroblast; CFU-Ob, CFU-osteoblast; Car2, carbonic anhydrase II; CTSK, cathepsin K; DFO, deferoxamine mesylate; FPN1, ferroportin 1; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HE, hematoxylin and eosin; Hepc, hepcidin-1; IR, ionizing radiation; μCT, micro-computed tomography; MAR, mineral apposition rate; MS/BS, mineralizing surface/bone surface; MMP9, matrix metallopeptidase 9; M-CSF, macrophage colony-stimulating factor; MSCs, mesenchymal stem cells; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; NF-κB, nuclear factor-kappa B; Ob.Pm/B.Pm, osteoblast perimeter/bone perimeter; Oc.Pm/B.Pm, osteoclast perimeter/bone perimeter; OCPs, Osteoclast progenitors; PBS, phosphate buffer saline; RANKL, receptor activator for nuclear factor-κ B ligand; SD, standard deviation; Tb.Th, trabecular thickness; Th.Sp, trabecular separation; Tb.N, trabecular number; TRAP, tartrate-resistant acid phosphatase; TfR1, transferrin receptor 1; Veh, vehicle

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1. Introduction

Radiation therapy is a highly targeted and effective modality in the clinical management of malignancies. Although significant improvements have been made with precise treatment planning and delivery, the area selected for irradiation usually includes the whole tumor plus the immediate surrounding normal tissue resulting in delayed side effects. Irradiation of the bone frequently leads to bone loss and increased risk of fractures [1], a condition commonly seen in patients with various cancers [2–5]. Furthermore, there is evidence of an abscopal effect in bone loss following single limb irradiation in that bone loss occurs not only at irradiated site, but at the contralateral, non-irradiated side as well [6–9]. The underlying mechanism is not well understood yet. Uncovering the mechanisms of both direct and indirect effects of radiation on bone are of great importance for developing preventive and curative treatment strategies.

Iron metabolism is closely related to bone homeostasis. Increased iron level contributes to bone loss, characterized by enhanced bone resorption and decreased bone formation. Osteoporosis frequently occurs in patients with hemochromatosis, African hemosiderosis, thalassemia or sickle cell disease that are associated with systemic iron overload [10]. There is evidence that an increase in total body iron storage is associated with high rate of bone loss in healthy postmenopausal women and middle-aged men [11]. These data suggest that enhanced iron storage could be an independent risk factor for accelerated bone loss, even in healthy populations [11]. Hepcidin, secreted by liver, is a key hormone that regulates iron homeostasis in the body. Hepcidin acts to lower iron in the blood by binding to and degrading the export protein ferroportin 1 (FPN1) [12]. Deficiency in hepcidin causes iron overload, which in turn upregulates hepcidin expression in a feedback mechanism [13]. Recent studies demonstrate that total body irradiation immediately increases serum iron in mice in a dose-dependent manner, which could persist for three weeks or even longer [14,15]. It is not clear whether increased iron storage occurs in the body after focal bone irradiation and whether the process is mediated by altered hepcidin expression. Furthermore, the question whether increased serum iron contributes to the systemic bone loss following radiation remains unanswered.

Since single limb irradiation could induce systemic bone loss and radiation has been shown to increase iron storage, we posed the hypothesis that increased iron storage induced by focal ionizing radiation (IR) mediated the subsequent systemic bone loss. Earlier reports on systemic bone loss utilized mostly large irradiated area covering the whole or large regions of hind-limb [7–9], a scenario that cannot recapitulate focal radiotherapy. In this study, we used a Small Animal Image Guided Irradiation System to precisely irradiate the mid-shaft of left femur in male C57BL/6 mice with a single dose of 2 Gy. The radiation was delivered in a circle collimated field with a diameter of 5 mm. Therefore, focally irradiated areas included only the bone marrow cavity and cortical bone of the mid-shaft and avoided the direct damage to both the distal and proximal trabecular bone. In the present study, we reported microarchitectural deterioration and activated osteoclast differentiation in irradiated and out of field, contralateral distal femurs. The systemic bone loss was associated with an abscopal effect on the liver resulting in elevated serum iron levels through down-regulation of hepcidin. Notably, IR directly stimulated local bone loss in irradiated bone. We further demonstrated in an animal model that lowering iron levels by injecting iron chelator, deferoxamine mesylate (DFO), and hepcidin 1 offered protection against IR-induced either systemic or local bone loss.

2. Material and methods

2.1. Animal study design

All animal studies described in this article were approved by the Animal Ethics and Welfare Committee at Soochow University. 4-week-old male C57BL/6J mice were purchased from and raised in laboratory animal center of Soochow University. The mice were fed with a standard pellet diet and distilled water ad libitum. Mice were group housed under standard vivarium with a 12 h light-dark cycle, at a temperature of 23 °C–25 °C and a relative humidity of 40 ± 5%. After acclimation, a Small Animal Image Guided Irradiation System (Precision X-Ray, North Branford, CT, USA) were used to precisely irradiate the mid-shaft of left femur at a single dose of 2 Gy on day 1. This system offers integrated precision irradiation with cone beam CT guidance and treatment planning systems with dose calculation tools based on Monte Carlo methods. Radiation was delivered in a circle collimated field with a diameter of 5 mm at a rate of 0.23 Gy/s. Control mice were similarly manipulated, and underwent cone beam CT scan. A single 2 Gy dose was used in the present study to simulate clinical radiotherapy setting consistent with previous studies on radiation-induced bone loss [7,16].

DFO (250 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and hepcidin-1 (500 μg/kg; Bachem AG, Bubendorf, Switzerland) or an equivalent volume of saline were injected intraperitoneally every other day. The subcutaneous injections started 2 days before irradiation and continued until the animals were sacrificed 28 days after radiation. The mice without DFO or hepcidin-1 treatment were sacrificed one week and four weeks after irradiation, respectively.

2.2. Micro-computed tomography (μCT) analysis

One and four weeks after radiation, femurs were harvested for μCT analyses (Skycan-1174, Bruker, Kontich, Belgium). Trabecular bone microarchitecture was evaluated in the distal metaphysis of the femur in a region that began 0.5 mm proximal to the growth plate and extended proximally 1 mm. Geometric trabecular analysis included bone mineral density (BMD), bone volume fraction (BV/TV), bone surface (BS), trabecular thickness (Tb.Th), trabecular separation (Th.Sp), and trabecular number (Tb.N). These parameters were calculated in accordance with guidelines for use of μCT in rodents [17].

2.3. Dynamic histomorphometry

Mice were injected subcutaneously with calcein (35 mg/kg; Sigma-Aldrich) 10 days and 3 days, respectively, before dissection. 28 days after irradiation, femurs were harvested, fixed in 75% ethanol, and then embedded with methyl methacrylate. Longitudinal sections were cut at 8 μm thickness for dynamic measurements. Pictures were taken using a fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany). Image J software (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/) was used to measure and analyze the following dynamic parameters: mineral apposition rate (MAR; μm/day), mineralizing surface/bone surface (MS/BS; %), and bone formation rate/bone surface (BFR/BS; mm^3/mm^2/day).

2.4. Histology

Bilateral femurs were harvested 4 weeks after radiation for histological analysis. Briefly, the femurs were fixed in 4% paraformaldehyde for 2 days, decalcified in 10% EDTA for 21 days, and processed for paraffin sections. The sections were stained with hematoxylin and eosin (H&E), and with tartrate-resistant acid phosphatase (TRAP) stain. The osteoblast perimeter (Ob.Pm/B.Pm) and osteoclast perimeter (Oc.Pm/B.Pm) were quantified relative to the bone surface. The sections were viewed on a light microscope (Leica Microsystems GmbH) and analyzed by ImageJ software (National Institutes of Health).

2.5. CFU-F and CFU-Ob assays for bone marrow mesenchymal stem cells

Bilateral femurs were harvested one day after irradiation. Bone marrow cells of medullary cavities were collected individually from