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Iron overload inhibits osteogenic commitment and differentiation of mesenchymal stem cells via the induction of ferritin

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ABSTRACT

Osteogenic differentiation of multipotent mesenchymal stem cells (MSCs) plays a crucial role in bone remodeling. Numerous studies have described the deleterious effect of iron overload on bone density and microarchitecture. Excess iron decreases osteoblast activity, leading to impaired extracellular matrix (ECM) mineralization. Additionally, iron overload facilitates osteoclast differentiation and bone resorption. These processes contribute to iron overload-associated bone loss. In this study we investigated the effect of iron on osteogenic differentiation of human bone marrow MSCs (BMSCs), the third player in bone remodeling.

We induced osteogenic differentiation of BMSCs in the presence or absence of iron (0–50 μmol/L) and examined ECM mineralization, Ca content of the ECM, mRNA and protein expressions of the osteogenic transcription factor runt-related transcription factor 2 (Runx2), and its targets osteocalcin (OCN) and alkaline phosphatase (ALP). Iron dose-dependently attenuated ECM mineralization and decreased the expressions of Runx2 and OCN. Iron accomplished complete inhibition of osteogenic differentiation of BMSCs at 50 μmol/L concentration. We demonstrated that in response to iron BMSCs upregulated the expression of ferritin. Administration of exogenous ferritin mimicked the anti-osteogenic effect of iron, and blocked the upregulation of Runx2, OCN and ALP. Iron overload in mice was associated with elevated ferritin and decreased Runx2 mRNA levels in compact bone osteoprogenitor cells. The inhibitory effect of iron is specific toward osteogenic differentiation of MSCs as neither chondrogenesis nor adipogenesis were influenced by excess iron. We concluded that iron and ferritin specifically inhibit osteogenic commitment and differentiation of BMSCs both in vitro and in vivo.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells, with the capability to differentiate into various mesenchymal lineages, including but not limited to osteoblasts, chondrocytes and adipocytes [1]. Numerous signaling cascades were explored driving the commitment, and differentiation of MSCs toward the different lineages [2].

Osteogenic differentiation of MSCs is driven by many secreted differentiation factors, including transforming growth factor-beta 1, fibroblast growth factor, bone morphogenetic protein, wingless proteins, Indian hedgehog, which all converge on the master osteogenic transcription factor, runt-related transcription factor 2 (Runx2) [3]. The crucial importance of Runx2 in osteogenic differentiation is highlighted by the finding, that Runx2 deficient mice completely lack differentiated osteoblasts resulting impaired bone formation, and die shortly after birth [4,5]. Runx2 regulates the transcription of the major bone-specific proteins, such as osteocalcin (OCN), osteopontin, bone sialoprotein, α1 type I collagen and alkaline phosphatase (ALP), those expression is required for proper bone formation [6]. Recently, the role of reactive oxygen species (ROS) in osteogenesis, as a common denominator of the diverse osteogenic signaling pathways, has been established [7]. Studies revealed that tightly regulated levels of ROS are critical in osteogenic differentiation of MSCs [7].

Hemochromatosis, the accumulation of excess iron in the body tissues, damages different organs, particularly the liver, the pancreas and the heart. Several reports described low bone density, osteoporosis and/or osteopenia in patients with different forms of hemochromatosis

Abbreviations: ALP, alkaline phosphatase; BMP2, bone morphogenetic protein 2; BMSCs, bone marrow-derived mesenchymal stem cells; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein di-acetate, acetyl ester; ECM, extracellular matrix; Fabp4, fatty acid-binding protein 4; FtH, ferritin heavy chain; FtL, ferritin light chain; HBSS, Hank's balanced salt solution; HPRT, hypoxanthine-guanine phosphoribosyl transferase; MSCs, mesenchymal stem cells; OCN, osteocalcin; OPG, osteoprotegerin; OPCs, osteoprogenitor cells; Pi, inorganic phosphate; RANKL, receptor activator of nuclear factor-kappa B ligand; ROS, reactive oxygen species; Runx2, runt-related transcription factor 2; VSMCs, vascular smooth muscle cells.

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[8–11]. Supporting this notion, both pharmacological and genetic provocation of iron overload was shown to be associated with bone abnormalities in rodent models of hemochromatosis. For example, intraperitoneal injection of ferric iron results in bone loss [12,13]. Additionally, a bone phenotype of osteoporosis with low bone mass and alteration of the bone microarchitecture has been described in a mouse model of genetic hemochromatosis [14]. Recently, hepcidin, the liver-derived regulator of iron homeostasis has been also identified as a factor influencing bone physiology. Lack of hepcidin triggers severe tissue iron overload that is associated with low bone mass, and altered bone microarchitecture [15,16].

Excess iron generates oxidative stress, characterized by an increase in the steady state level of ROS, which mediates cellular and tissue damage, that is believed to be the major pathogenic factor in iron overload diseases [17]. To counteract with iron toxicity, excess iron is sequestered by ferritin, the major iron storage molecule. Ferritin shell is made up of 24 subunits of two types, heavy (H) and light (L), and can accommodate as much as 4500 Fe^{3+} ions in a safe and bioavailable form [18,19]. The H subunit exhibits ferroxidase activity that promotes oxidation of Fe^{2+} into Fe^{3+}, while the L subunit plays a role in iron nucleation, and long-term iron storage [18,20]. The expression of ferritin subunits are tightly regulated by iron, both the transcriptional and translational levels [21–23].

Bone is an active tissue that is continuously being reshaped, in a process called remodeling, in which osteoclasts resorb bone tissue, whereas osteoblasts deposit new bone tissue. The cellular mechanism behind the detrimental role of excess iron in bone physiology can be the consequence of facilitated differentiation, and increased activity of osteoclasts, or decreased activity of osteoblasts, or both. Iron has been shown to facilitate osteoclast differentiation [24]. Also, osteoclast activity is increased in thalassemia major patients, but this was found to be rather associated with inflammation, than increased iron stores [25]. Previously, we showed that iron potently inhibited matrix mineralization of mature osteoblasts [26]. Iron-mediated inhibition of osteoblast matrix mineralization relied on decreased phosphate uptake, and the downregulation of Runx2, the osteoblast-specific transcription factor and its target transcripts: OCN and ALP [26]. Ferritin, the iron-regulated, iron-storage protein, mimicked the inhibitory effect of iron on matrix mineralization of mature osteoblasts [26].

Osteoblasts derive from mesenchymal progenitor cells, which migrate to the site of injury, proliferate and differentiate [27]. Osteogenic potential of these bone marrow-derived MSCs (BMSCs) is of crucial importance for proper remodeling and/or healing, which is highlighted by the recent discovery, that osteoporosis is associated with low osteogenic potential of circulating MSCs [28]. This prompted us to examine the role of iron in MSC osteogenic commitment, and differentiation.

2. Materials and methods

2.1. Materials

Unless otherwise specified all reagents were from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Cell culture

Human BMSCs derived from 2 different adult donors of Caucasian origin were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were maintained in DMEM (high glucose), containing 10% FBS (Gibco, Grand Island, NY, USA), 10,000 U/mL penicillin, 10 mg/mL streptomycin, 25 μg/mL amphotericin B, and 1 mmol/L sodium pyruvate. Cells were grown to confluence, and used from passages 4 through 7. Iron was introduced as ferrous sulfate. Ferritin was introduced as holo-ferritin (Ft).

2.3. Mice

All experiments were performed in compliance with institutional (Institutional Ethics Committee, University of Debrecen) and national guidelines. Ten C57BL/6 mice (8–10 weeks old, male) were randomly divided into two groups. The iron-overload group was injected intraperitoneally with iron-dextran (200 mg/kg body weight, 3-times, every other day), while control mice were treated with PBS. Mice were sacrificed with CO_2 inhalation and perfused with 5 mL of ice-cold PBS, tibia and femur were harvested for analysis.

2.4. Isolation of compact bone osteoprogenitor cells (OPCs)

OPCs were isolated from compact bones as described by Zhu et al. [29]. Briefly, after harvesting and cleaning the tibia and femur, the epiphyses were removed. To release bone marrow, bones were fragmented and agitated in a mortar containing ice cold PBS supplemented with 2% FBS and 1 mmol/L EDTA. This washing step was repeated 5 times, and the bone-marrow free bones were digested in 0.25% Collagenase Type I (5 min, RT). Then the bones were chopped into 1–2 mm pieces and digested for an additional 45 min at 37 °C. Finally the bone chips were washed, and the obtained suspension was filtered through a 70 μm cell strainer and centrifuged (300*g*, 10 min, RT). Harvested cells were used to isolate RNA immediately.

2.5. Induction of osteogenic differentiation

At confluence, BMSCs were switched to osteogenic medium, which was prepared by adding inorganic phosphate (Pi) (0–3 mmol/L) and Ca at the form of $CaCl_2$ (0–1.2 mmol/L) to the growth medium. Both growth medium and osteogenic medium were changed in every other day. Unless otherwise specified, we used an osteogenic medium that was supplemented with 3 mmol/L Pi and 1.2 mmol/L Ca. Alternatively, osteogenic differentiation was induced by dexamethasone (0.1 μmol/L), ascorbic acid (50 μmol/L) and β-glycerol-phosphate (2 mmol/L) for 14 days. When appropriate, cycloheximide (CHX) was used to inhibit protein synthesis at a concentration of 10 μg/mL.

2.6. Induction of chondrogenic differentiation

To induce chondrogenic differentiation, BMSCs were cultured in chondrogenic medium, containing StemPro Chondrogenesis Supplement (Gibco, Grand Island, NY, USA) and gentamicin (10 mg/mL) for 14 days.

2.7. Induction of adipogenic differentiation

To induce adipogenic differentiation, confluent BMSCs were cultured in adipocyte differentiation medium, containing StemPro Adipocyte Supplement (Gibco, Grand Island, NY, USA) and gentamicin (10 mg/mL) for 3 weeks.

2.8. Alizarin Red staining

After washing with PBS, the cells were fixed in 4% paraformaldehyde and rinsed with deionized water thoroughly. Cells were stained with Alizarin Red S solution (2%, pH 4.2) for 20 min at room temperature. Excessive dye was removed by several washes in deionized water. Extracellular Ca deposition was stained in red color using Alizarin Red S dye.

2.9. Alcian blue staining

Alcian blue staining was performed with NovaUltra Special Stain Kit (IHC World, Ellicott City, MD, USA) according to the manufacturer's instructions.