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Mice with increased angiogenesis and osteogenesis due to conditional activation of HIF pathway in osteoblasts are protected from ovariectomy induced bone loss

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ABSTRACT

Postmenopausal osteoporosis is characterized by a reduction in the numbers of sinusoidal and arterial capillaries in the bone marrow and reduced bone perfusion suggesting a role of vascular component in the pathogenesis of osteoporosis. Previous studies have shown that bone formation and angiogenesis are positively coupled through activation of the hypoxia inducible factor (HIF1α) signaling pathway. Therefore, we hypothesized that mice with increased angiogenesis and osteogenesis due to activation of the HIF signaling pathway in osteoblasts, via osteoblast specific disruption of HIF degrading protein von Hippel–Lindau (VHL) (ΔVhl), are protected from ovariectomy induced bone loss. ΔVhl mice and control littermates were ovariectomized or sham operated and four weeks later bone quality was evaluated along with blood vessel formation. Trabecular and cortical bone volume was strikingly increased in ΔVhl mice along with blood vessel formation as compared to control littermates. In control mice, ovariectomy significantly decreased bone mineral density, deteriorated bone microarchitecture, and decreased mechanical strength compared to the sham operated control mice. This was accompanied with a significant decrease in blood vessel volume and expressions of HIF1α, HIF2α, and VEGF proteins at the distal femur in ovariectomized control mice. In contrast, ovariectomy in ΔVhl mice had absolutely no effect on either the blood vessel formation or the bone structural and mechanical quality parameters. These data indicate that activation of HIF signaling pathway in osteoblasts may prevent estrogen deficiency-induced bone loss and decrease in blood vessels in bone marrow.

Introduction

Osteoporosis is a skeletal disease characterized by low bone mass and microarchitectural deterioration with a resulting increase in bone fragility and subsequent susceptibility to fractures [1]. The prevalence of osteoporosis increases with increasing age in both men and women with postmenopausal women as a very high risk group [2]. Although the primary cause of postmenopausal osteoporosis is estrogen deficiency [3], it is also characterized by a reduction in the number of sinusoidal and arterial capillaries in the bone marrow and reduced bone perfusion [4–6] suggesting a role of vascular component in the pathogenesis of postmenopausal osteoporosis. The skeleton is a highly vascularized tissue that receives 5–20% of cardiac output [7]. Developmental studies show that a close coupling between angiogenesis and osteogenesis is essential for normal bone formation [8]. Vascular invasion into cartilage anlagen is essential for long bone formation during endochondral ossification [9]. Moreover, disorders in bone vasculature can affect skeletal homeostasis. For example, impairment of angiogenesis decreases trabecular bone formation as well as the expansion of the hypertrophic zone into the growth plate [10], and surgical interruption of the blood supply to bone results in marked decrease in bone density and strength [11].

On molecular level, developing vasculature and its maintenance is guided by oxygen-gradient and hypoxia-inducible factors (HIFs) [12]. The HIF signaling pathway is also a central regulator of osteogenesis and angiogenesis coupling [13]. The HIF family consists of 3 α subunits, HIF1α, HIF2α, and HIF3α, which form a heterodimer complex with the HIF1β subunit. HIF1β is constitutively expressed whereas HIF-α is an oxygen-labile protein. The α-subunit has an oxygen-dependent degradation domain (ODD) which is recognized by prolyl hydroxylase domain (PHD) proteins. Under normoxic circumstances, PHD proteins hydroxylate HIFα and form a complex with von Hippel–Lindau protein (pVHL), a E3 ubiquitin ligase, that primes HIFα for proteosomal degradation [14]. During hypoxic conditions, prolyl hydroxylation of the α subunit is blocked and HIFα protein accumulates in cytoplasm. It then freely translocates to the nucleus where it heterodimerizes with the β subunit and initiates HIF-responsive genes transcription [15]. Absence of pVHL, and hence the lack of proteosomal degradation of HIFα leads to accumulation

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of HIFα in the cytoplasm [13,16,17] and represents a mechanism to activate HIFα signaling. In-vivo conditional activation of HIFα signaling pathway in osteoblasts by osteoblast-specific deletion of Vhl (ΔVHL) results not only in a dramatic increase in bone mass but also increases the vasculature in bone marrow cavity [13]. Conversely, mice lacking HIF-1α and/or HIF-2α in osteoblasts (ΔHIF) showed a significant decrease in bone volume as well as blood vessel formation [13]. These data reveal a role of HIF signaling in osteoblasts in maintaining bone homeostasis and also in promoting skeletal vascularization. The progressive increase in bone volume in ΔVHL mice results from increased numbers and activity of osteoblasts whereas osteopenia in the ΔHIF is associated with a significant decrease in osteoblast numbers and osteoid volume. However, disruption of Vhl in-vitro does not affect osteoblast proliferation or function indicating that HIF-1α regulates bone formation via cell nonautonomous mechanism. In fact, the amount of bone in these mutant mice is directly proportional to the amount of skeletal vascularity which is proportional to the increased secretion of VEGF by osteoblasts, a downstream target of HIFα signaling.

In this study, we examined whether the skeletal vascularization is decreased in ovariectomized (OVX) mice and whether increasing skeletal blood vessel formation by activation of HIF signaling in osteoblasts can prevent ovariectomy induced bone loss. To answer this question, we used ovariectomized mice with conditional activation of HIFα signaling pathway in osteoblasts by osteoblast-specific deletion of Vhl (ΔVhl) and compared their bone phenotype with normal OVX mice. We have found that skeletal vascularization and bone volume are indeed decreased in OVX mice and the bone loss can be prevented by upregulation of HIF signaling as in ΔVhl mice.

Materials and methods

Animal model

All procedures involving mice were approved by the Shanghai Jiaotong University Animal Study Committee and were carried out in accordance with the guide for the humane use and care of laboratory animals. Mice with osteoblast-specific inactivation of Vhl (ΔVhl) were generated by intercrossing osteocalcin-Cre (OC-Cre) transgenic mice [18] with mice homozygous for floxed Vhl allele [19] (both mice kindly provided by Dr. Thomas L. Clemens, Department of Orthopaedic Surgery, Johns Hopkins University School of Medicine, Baltimore, MD). Littermates were used as controls for all experiments. Genotyping for OC-Cre and Vhl alleles were performed as described previously [13]. Nine-week-old female control and ΔVhl mice were randomly assigned in equal numbers to sham and ovariectomy operation groups. The animals were housed 5 per cage and were maintained under a strict 12 h light:12 h darkness cycle at 22 °C with standard mice food pellets and had free access to tap water. After anesthetization, control and ΔVhl mice were ovariectomized (OVX), or sham-operated (Sham). Both ovaries were exposed and removed in the OVX animals. While in the Sham animals, the ovaries were exposed but left intact. At the end of experimental period, the mice were sacrificed by overdosing of anesthetics. Uteri of the mice were isolated and weighed to confirm the effects of ovariectomy. Body weights of sham-operated and OVX mice in both genotypes were recorded weekly to investigate the effect of body weight on bone.

Skeletal phenotyping and histological analysis

The distal end of intact right femurs from control and ΔVhl mice were scanned using μCT (GE Locus SP) to assess bone mass, density, geometry, and trabecular microarchitecture. Parameters computed from these data include bone mineral density (BMD), bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp). For histological analysis of bone, the right tibiae were dissected and fixed in 4% paraformaldehyde at 4 °C for 48 h, and then were decalcified in 10% EDTA for about 4 weeks. Bones were then dehydrated in increasing concentrations of ethanol, cleared in xylene, and paraffin-embedded and serial 5 μm sagittal sections were cut using a Leica microtome (Leica RM 2135, Germany). The sections were then stained with Hematoxylin–Eosin (H&E) staining and trabecular bone and marrow cavity at proximal tibia were examined using 20× objective lens connected to a video camera (ZEISS, AXIO). Uterus tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5 μm transverse sections, and stained with H&E for morphological examination.

Immunohistochemistry

For immunohistochemistry, decalcificated gelatinized tibiae sections were boiled in 10 mM sodium citrate (pH 6.0) for 5 min to retrieve antigen. Sections were quenched with 3% hydrogen peroxide for 15 min to reduce endogenous peroxides activity and blocked with 3% normal goat serum in Tris-buffered saline. The sections were then incubated with rabbit anti-mouse HIF-1α, HIF-2α and VEGF polyclonal antibodies (Santa Cruz Biotechnology, CA) at 4 °C overnight, followed by biotinylated secondary antibodies and a peroxidase-labeled streptavidin–biotin staining technique (DAB kit, Invitrogen). Nuclei were counterstained with hemalum (FARCO Chemical Supplies, Hong Kong). The slides were visualized by a microscope (ZEISS, AXIO). The slides without incubation with secondary antibody were used as negative controls.

Real time PCR analysis

Epiphyses removed and bone marrow depleted tibiae from 4-week post-sham and OVX operated control mice were homogenized in Trizol (Invitrogen) using a tissue homogenizer and RNA was then extracted as per the manufacturer's recommendations. One microgram RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad), and amplified by real-time PCR using SYBR GREEN PCR Master Mix (Applied Biosystems) and primers for Hif-1α, Hif-2α and Vegf. The primers used were: HIF1α (Forward 5′-GAAGACAACGCGGGCACCGA-3′; Reverse 5′-TGCTTCGCCGAGATCTTGCTGC-3′), HIF-2α (Forward 5′-CGGGGCCCGAGGTCCATACA-3′; Reverse 5′-CATCGGGGGCCATGTTCGCA-3′), Vegf (Forward 5′-CCCGGGCCTCGGTTCCAG-3′; Reverse 5′-GTCGTGGGTGCAGCCTGGG-3′), and β-actin (Forward 5′-TTCGTTGCCGGTCCACACCC-3′; Reverse 5′-GCTTTGCACATGCCGGAGCC-3′).

Mechanical testing

The femurs from control and ΔVhl mice were flash frozen in liquid nitrogen immediately after sacrificing the mice and stored in −80 °C for further mechanical testing. The femurs were thawed at room temperature before testing and three-point bending of the right femora was carried out by an Instron 5569 materials testing machine (Instron Inc., MA). The femur was placed posterior side down between two supports which were 6 mm apart and load was applied at the mid-span which made bending occur about the anteroposterior axis. Load–displacement curves were recorded at a crosshead speed of 1 mm/s.

Imaging of blood vessels

Specimens were prepared in accordance with previously described methods [20]. Briefly, after animals were euthanized, the thoracic cavity was opened, and the inferior vena cava was severed. The vasculature was flushed with 0.9% normal saline containing heparin sodium (100 U/ml) at a pressure of approximately 100 mm Hg via a needle inserted into the left ventricle. The specimens were then pressure fixed with 10% neutral buffered formalin. Formalin