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ATP6v0d2 deficiency increases bone mass, but does not influence ovariectomy-induced bone loss

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ABSTRACT

Bone homeostasis is maintained through the balanced action of bone-forming osteoblasts and bone-resorbing osteoclasts. Under pathological conditions or with age, excessive bone loss is often observed due to increased bone resorption. Since osteoclasts are the primary cells in the body that can resorb bone, molecular understanding of osteoclast fate has important clinical implications. Over the past 20 years, many molecular players that govern osteoclast differentiation during normal development have been identified. However, whether the same molecules regulate bone loss occurring under pathological conditions remains largely unknown. We report here that although ATP6v0d2-deficient (ATP6v0d2 KO) mice exhibit an osteopetrotic phenotype due to inefficient osteoclast maturation, this deficiency fails to protect mice from ovariectomy (OVX)-induced bone loss, a model for post-menopause-associated osteoporosis. Moreover, we show that an OVX-induced increase in the number of colony forming unit-granulocyte/macrophage (CFU-GM) in bone marrow cells and subsequent osteoclast formation *in vitro* was not affected in the absence of ATP6v0d2. However, even after OVX, formation of large osteoclasts (>100 μm in diameter) with actin rings was still reduced in the absence of ATP6v0d2. Taken together, these findings suggest that the critical role of ATP6v0d2 may be limited to the control of bone homeostasis under normal development, and that OVX-induced bone loss is likely to be governed mostly by the increase in osteoclast precursors rather than increased efficiency of osteoclast maturation.

1. Introduction

Bone remodeling controls homeostasis of the skeletal systems and is maintained by a balance between bone matrix-producing osteoblasts and matrix-resorbing osteoclasts [1,2]. Mature osteoclasts are the only cells that efficiently resorb bone. They differentiate from hematopoietic precursors, which also comprise colony forming unit-granulocyte/macrophage (CFU-GM) cells [3,4]. Bone marrow precursors differentiate to $TRAP^+$ preosteoclasts, which are mononuclear, via receptor activator of the NF-κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) stimulation. These mononuclear preosteoclasts undergo cell–cell fusion to form multinucleated osteoclasts. Such multinucleated giant cell formation is critical for osteoclast maturation and efficient bone resorption [5,6]. In addition, the activity or viability of mature multinuclear osteoclasts can be further regulated by various stimulators including inflammatory cytokines [3,7]. Mature osteoclasts can resorb bone by secreting hydrogen ions and various acidic proteases, including TRAP and cathepsin K, into the resorption lacunae between the osteoclasts and the bone surface to facilitate the removal of inorganic and organic bone matrix, respectively [4]. Acidification of the lacunae is primarily mediated by vacuolar H^+-ATPase (v-ATPase), which is predominantly located in the ruffled border of osteoclasts [8,9].

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Previous studies have identified multiple isoforms of v-ATPase subunits, with distinct cell type- and tissue-specific expressions. These isoforms are proposed to account for the diverse physiological properties of v-ATPases, which are ubiquitous in all cell types [10,11]. It was previously reported that gene-targeted mice that are deficient in the a3 isoform of the v-ATPase (Atp6i) exhibited severe osteopetrosis due to defects in osteoclast-mediated extracellular acidification [12]. Furthermore, mutations in the a3 subunit in humans (known as TCIRG1) were responsible for infantile malignant osteopetrosis [13,14].

We previously reported [6], that mice lacking the d2 isoform of the v-ATPase V0 domain (ATP6v0d2) had an increased bone mass due to defective maturation of osteoclasts and enhanced bone formation by osteoblasts. However, ATP6v0d2 deficiency did not affect either the early differentiation or the v-ATPase activity of osteoclasts. Despite an increase in bone formation observed in ATP6v0d2 deficient mice *in vivo*, ATP6v0d2 mRNA was not detected in osteoblasts, and *ex vivo* osteoblast differentiation and mineralization did not seem to be affected in the absence of ATP6v0d2. Therefore, it seemed that increased bone formation was probably due to osteoblasts-extrinsic factors.

Estrogen deficiency, which induces marked increases in osteoclastic bone resorption, produces rapid bone loss in humans and experimental animals. Ovariectomized (OVX) animal models have been extensively used as an experimental model of enhanced bone resorption [15,16]. Previously, it was found that various cytokines, growth factors and free radicals, which were locally produced, stimulated osteoclastic bone resorption in an estrogen-deficient environment [17–19]. However, it is still largely unknown how estrogen deficiency causes increased number of osteoclasts and subsequent bone resorption *in vivo*.

In the present study, we investigated the effect of ATP6v0d2 on OVX-induced bone loss by measuring trabecular bone mass, osteoclast differentiation and CFU-GM formation in wild-type and ATP6v0d2 KO mice.

2. Materials and methods

2.1. Experimental animals

All experiments were performed on WT and ATP6v0d2 KO mice in a C57BL/6J background. The WT controls and homozygous ATP6v0d2 KO mice were generated by mating of heterozygous ATP6v0d2 KO littermates. Initially, ATP6v0d2 KO mice were produced in a mixed background of C57BL/6 and 129 SV. Later, these mice were backcrossed into C57BL/6 for at least 10 generations prior to the experiments. The WT and ATP6v0d2 KO mice were either sham-operated (SHAM) or OVX, and were euthanized 4 weeks later. OVX status was confirmed by measurement of uterine weight at the time of death. All mouse work was performed under veterinary supervision in an accredited facility using protocols approved by the Animal Care and Use Committee of the University of Pennsylvania School of Medicine and the Wonkwang University School of Dentistry.

2.2. Reagents

All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA, USA). Soluble recombinant mouse RANKL was purified from insect cells as previously described [20], and recombinant human M-CSF was the kind gift of David H. Fremont (Washington University, St. Louis, MO). GM-CSF was purchased from R&D Systems (Minneapolis, MN, USA) and other reagents were purchased from Sigma (St. Louis, MO, USA).

2.3. Bone analysis by μCT

Trabecular morphometry within the metaphyseal region of the distal femur was quantified using micro-CT (μCT40, Scanco Medical AG, Bassersdorf, Switzerland) as described [21]. μCT analysis was performed in the Center for Bone Histomorphometry at the University of Connecticut Health Center. Bones from 5 to 6 mice per group were examined. Three-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering, and were rendered at a discrete density of 578,704 voxels/mm^3 (isometric 12-μm voxels). A threshold segmentation of bone from marrow and soft tissue was performed in conjunction with a constrained Gaussian filter to reduce noise. Volumetric regions for trabecular analysis were selected within endosteal borders to include the central 80% of vertebral height, as well as the secondary spongiosa of femoral metaphyses located 960 μm (about 6% of length) from the growth plate. Trabecular morphometry was characterized by measuring bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp). Cortical morphometry was analyzed within a 600 μm-long section at the mid-diaphysis of the femur and included measurements of average thickness and cross-sectional area.

2.4. In vitro formation of osteoclast-like cells

Bone marrow cells from WT and ATP6v0d2 mice were isolated and allowed to differentiate into osteoclast-like cells (OCLs) as described previously [21,22]. Briefly, bone marrow cells from the femur and tibia were collected and washed with α-MEM. After washing, bone marrow cells were cultured (1.5×10^5 cells/well in 96-well culture plate) in α-MEM containing 10% FBS with RANKL (100 ng/ml) and M-CSF (50 ng/ml) for up to 4 days. Every 3 days, the medium was replaced with fresh medium containing RANKL and M-CSF. Cells were fixed with 10% formalin after 4 days, followed by TRAP staining. The F-actin ring was stained with rhodamine phalloidin (Molecular Probes) before TRAP staining. Any TRAP$^+$-multinuclear cells (MNCs) that measured more than 100 μm in diameter and contained more than three nuclei and actin ring were considered to be OLCs. In some experiments, total TRAP activity was measured at an absorbance of 405 nm after treatment with substrate (ρ-nitrophenyl phosphate) as described previously [6].

2.5. Colony forming unit-granulocyte/macrophage (CFU-GM) assay

Bone marrow cells from WT and ATP6v0d2 mice were prepared as described above. The CFU-GM assay was performed as previously described [22] with slight modification. Briefly, bone marrow cells (1×10^5 cells/ml/dish) were plated on a 35-mm tissue culture dish in 1 ml of 1.3% methylcellulose (MethoCultTMH4100; Stem Cell Technologies Inc.) supplemented with 30% FBS, 2% BSA (Sigma), 2 mM L-glutamine (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma) and 5 ng/ml GM-CSF (R&D Systems), as a source of colony-stimulating activity. Cultures were maintained at 37 °C for 6 days and colonies (>40 cells) were scored at the end of the incubation.

2.6. Statistical analysis

Statistical analysis for mouse bone was performed by one-way ANOVA and the Bonferroni posthoc test when ANOVA showed significant differences. Student's *t*-test was used when the others were compared. All experiments were repeated at least twice, and representative data are shown.