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Loss of RANKL in osteocytes dramatically increases cancellous bone mass in the osteogenesis imperfecta mouse (oim)

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ABSTRACT

Osteogenesis imperfecta (OI) is characterized by osteopenia and bone fragility, and OI patients during growth often exhibit high bone turnover with the net result of low bone mass. Recent evidence shows that osteocytes significantly affect bone remodeling under physiological and pathological conditions through production of osteoclastogenic cytokines. The receptor activator of nuclear factor kappa-B ligand (RANKL) produced by osteocytes for example, is a critical mediator of bone loss caused by ovariectomy, low-calcium diet, unloading and glucocorticoid treatment. Because OI bone has increased density of osteocytes and these cells are embedded in matrix with abnormal type I collagen, we hypothesized that osteocyte-derived RANKL contributes to the OI bone phenotype. In this study, the conditional loss of RANKL in osteocytes in oim/oim mice (oim-RANKL-cKO) resulted in dramatically increased cancellous bone mass in both the femur and lumbar spine compared to oim/oim mice. Bone cortical thickness increased significantly only in spine but ultimate bone strength in the long bone and spine was minimally improved in oim-RANKL-cKO mice compared to oim/oim mice. Furthermore, unlike previous findings, we report that oim/oim mice do not exhibit high bone turnover suggesting that their low bone mass is likely due to defective bone formation and not increased bone resorption. The loss of osteocyte-derived RANKL further diminished parameters of formation in oim-RANKL-cKO. Our results indicate that osteocytes contribute significantly to the low bone mass observed in OI and the effect of loss of RANKL from these cells is similar to its systemic inhibition.

1. Introduction

Osteogenesis imperfecta (OI) is the most common congenital skeletal fragility disorder, affecting approximately 6–7 per 100,000 people worldwide (Genetics Home Reference, n.d.). Also known as brittle bone disease, OI is mainly characterized by fragile bones and low bone mass, and most cases (around 85%) are caused by dominant mutations in the type I collagen genes, *COL1A1* or *COL1A2* (*Marini* et al., *2017*). At the bone tissue level, the disease is generally characterized by low bone mass and a high fracture rate, with significant alterations in bone modeling and endochondral ossification, and bone remodeling (Rauch et al., 2000). At the cellular level, osteoblasts, the bone forming cells, and their function have been the main focus of research in OI pathogenesis. This is justified by the fact that osteoblasts are the key producers of bone matrix (osteoid) whose main component is type I collagen. A defective type I collagen can dramatically impact bone matrix quality and its proper mineralization, and when incorrectly folded can also be retained intracellularly and cause ER stress, ultimately resulting in dysfunctional osteoblasts that deposit less bone which is also brittle. Osteoclasts are likely indirectly affected by a defective type I collagen and are often present in higher number on OI bone surfaces compared to healthy controls as shown by a bone histomorphometric study of a pediatric cohort of patients with OI type I, III and IV (Rauch et al., 2000). This study showed a marked increase in all bone surface-based formation and resorption indices, indicating a state of high bone turnover which was also reported by others (Rauch et al.,

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2000; Baron et al., 1983; Braga et al., 2004). Interestingly, while the amount of bone turned over in each remodeling cycle appears to be decreased in OI, the activation frequency, defined as the number of remodeling units per bone surface per time unit, is markedly elevated (up to 60% more in OI type I compared to controls) (Rauch et al., 2000). The third type of bone cells, the osteocytes, are long-lived compared to osteoblasts and osteoclasts and are the most numerous cells in the adult skeleton. Studies showed that osteocytes affect osteoblast and osteoclast formation and function through the local synthesis and secretion of growth factors and cytokines such as WNT ligands and Sclerostin, but also RANKL, an essential cytokine for osteoclastogenesis, and osteoprotegerin (OPG), an inhibitor of RANKL signaling (Bellido, 2014; van Bezooijen et al., 2004; Joeng et al., 2017). Importantly, it was recently shown that osteocytes are the most important source of RANKL during bone remodeling (Xiong et al., 2011; Nakashima et al., 2011) and osteocyte-derived RANKL is a critical mediator of the increased bone resorption and bone loss caused by ovariectomy, low-calcium diet, unloading and glucocorticoid treatment (Xiong et al., 2014; O'Brien et al., 2013; Fujiwara et al., 2016; Piemontese et al., 2016). It is known that OI bone exhibits increased density of osteocytes which are embedded in a matrix of altered type I collagen, however how the dysregulation/activity of these cells may potentially contribute to the OI disease phenotype is currently understudied. Because current treatments for OI also aim to decrease bone resorption (for instance using bisphosphonates or the anti-RANKL monoclonal antibody Denosumab), these could be made more effective through a better understanding of the underlying cellular mechanisms.

In this study, we generated a novel mouse model to test our hypothesis that RANKL from osteocytes contributes significantly to the low bone mass and the high bone turnover observed in OI. To do this we mated oim mice ($Col1a2^{oim/oim}$), a widely accepted mouse model of moderate to severe OI (Chipman et al., 1993), with mice carrying a floxed *Tnfsf11* gene (encodes RANKL) and a Cre transgene driven by the *Dmp1* promoter (Xiong et al., 2011; Lu et al., 2007). This mouse (called "oim-RANKL-cKO") is a model for OI but lacks RANKL expression in osteocytes, and therefore enabled us to determine the contribution of osteocyte-produced RANKL to the skeletal phenotype of the oim mouse model.

2. Materials & methods

2.1. Mouse generation and genotyping

The use of laboratory mice was approved by the University of Arkansas for Medical Sciences (UAMS) IACUC committee. Mice were housed in a pathogen free facility with 12-hour light/dark cycle with unlimited access to water and standard chow diet. RANKL-cKO mice ($Tnfsf11l^{Fl/Fl}$; *Dmp1-Cre*) were generated with loxP sites flanking exons 3 and 4 of *Tnfsf11* and a Cre recombinase driven by a construct containing 9.6 kb of the *Dmp1* promoter and 4 kb of *Dmp1* exon 1 on a C57Bl/6 background by Dr. Charles O'Brien (Xiong et al., 2011; Lu et al., 2007). The oim mice ($Col1a2^{oim/+}$) in a pure C57Bl/6 genetic background were obtained from Dr. Charlotte Phillips at the University of Missouri-Columbia, MO (Carleton et al., 2008). The oim mice were maintained in a separate colony and also bred with the RANKL-cKO mice to produce mice with the genotype of interest in three steps, keeping the mice heterozygous for the oim mutation until the final step and always hemizygous for the Cre allele (for schematic see Fig. 1A). The mice were weighed once every week beginning at 5 weeks of age until sacrifice at 13 weeks of age. At sacrifice, the number of femora and tibiae per mouse with fractures were recorded. PCR genotyping was performed with the GoTaq G2 Hot Start Polymerase reagent (cat# M7423 Promega) and a Master Cycler thermocycler (Eppendorf). Protocols for *Rankl-flox* and *Dmp1-Cre* genotyping were described previously (Xiong et al., 2011). To ensure the correct conditional knockout of RANKL, a new genotyping protocol was developed to detect the deletion. Utilizing the primers Forward 5′-CTGGGAGCGCAGGTTAA ATA-3′ (the same forward primer as for *Tnfsf11-flox* genotyping) and Reverse 5′-GAGACATTAGAGCCCGGTCA-3′, this protocol amplifies the genomic DNA region encompassing the entire floxed region of *Tnfsf11* plus approx. 800 bp downstream of that region (Supplementary Fig. 1A). The products of the amplification are a large band (approx. 2.1 kb) for the intact *Tnfsf11-flox* allele or a smaller band (approx. 800 bp) for the recombined *Tnfsf11* allele (Supplementary Fig. 1B).

A new genotyping strategy was developed for detecting the oim mutation, based on the T-ARMS PCR method (Ye et al., 2001). The primers are 5′-ACTGTCTGTCTACAGTGAACGTCTTAA T-3′ outer forward, 5′-GATGTAGATGCATAGAAGACATGGAAGG-3′ outer reverse, 5′-TTCCCATTTTTTTCTATTATACAGAAACAG-3′ inner forward (WT specific), and 5′-AATGATTGTCTTGCCCCATTCATTTTTT-3′ inner reverse (oim specific) which flank the single nucleotide deletion (Supplementary Fig. 1C). These four primers were added to the same master mix for a final concentration of 0.1 μM (outer primers) and 1.0 μM (inner primers). The PCR program is as follows: 94 °C for 2 min, then 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and lastly, 72 °C for 5 min. The products are 440 bp (all genotypes), 303 bp (WT allele) and 195 bp (oim allele) and an example gel image of the results is provided in Supplementary Fig. 1D.

2.2. Micro-CT

Femur and lumbar spine were harvested from 3 month old male and female oim-RANKL-cKO mice and control groups and fixed in 95% ethanol. Micro-CT analysis was performed on a Micro-CT 40 (Scanco Medical AG, Bassersdorf, Switzerland) using a 12 μm isotropic voxel size. For cancellous bone of the femur, the region of interest selected for analysis comprised 200 transverse CT slices representing the entire medullary volume extending 1.24 mm distal to the end of the primary spongiosa with a border lying 100 μm from the cortex. For cancellous bone of the L4 vertebral body, the region of interest for analysis comprised the entire cancellous bone volume. Three-dimensional reconstructions were created by stacking the regions of interest from each two-dimensional slice and then applying a grayscale threshold and Gaussian noise filter (sigma 0.8, support 1, threshold 245) as described (Suva et al., 2008), using a consistent and pre-determined threshold with all data acquired at 55 kVp, 114 mA, and 200 ms integration time. Fractional bone volume (bone volume/tissue volume; BV/TV) and architectural properties of trabecular bone such as trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, mm^{-1}), and connectivity density (Conn. D, mm^{-3}) were calculated using previously published methods (Suva et al., 2008). Femoral cortical geometry was assessed in a region centered at the femoral midshaft. The outer surface of the bone was found automatically using the manufacturer's built-in contouring tool. Total area was calculated by counting all voxels within the contour and bone area by counting all voxels that were segmented as bone. Marrow area was calculated as total area minus bone area. This calculation was performed on all 50 slices (1 slice = 12.5 μm), using the average for the final calculation. The outer and inner perimeter of the cortical midshaft was determined by a three-dimensional triangulation of the bone surface (BS); other cortical parameters were determined as described (Suva et al., 2008). Vertebral cortical bone measurements were made on the ventral side of the L4 vertebral body, encompassing 100 slices from the distal growth plate, or the maximum slices possible between one growth plate and the other. The reporting of micro-CT measurements follows the guidelines of Bouxsein et al. (Bouxsein et al., 2010). Femur length was measured using a digital caliper (Whitworth). Each sample was measured twice and the average of the two measurements was used for data analysis.

2.3. Biomechanics

Femurs from 3 month old male and female oim-RANKL-cKO mice