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Glucocorticoids cause mandibular bone fragility and suppress osteocyte perilacunar-canalicular remodeling

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ABSTRACT

Osteocytes support dynamic, cell-intrinsic resorption and deposition of bone matrix through a process called perilacunar/canalicular remodeling (PLR). In long bones, PLR depends on MMP13 and is tightly regulated by PTH, sclerostin, TGFβ, and glucocorticoids. However, PLR is regulated differently in the cochlea, suggesting a mechanism that is anatomically distinct. Unlike long bones, the mandible derives from neural crest and exhibits unique susceptibility to medication and radiation induced osteonecrosis. Therefore, we sought to determine if PLR in the mandible is suppressed by glucocorticoids, as it is in long bone. Hemimandibles were collected from mice subcutaneously implanted with prednisolone or vehicle containing pellets for 7, 21, or 55 days (n = 8/group) for radiographic and histological analyses. Within 21 days, micro-computed tomography revealed a glucocorticoid-dependent reduction in bone volume/total volume and trabecular thickness and a significant decrease in bone mineral density after 55 days. Within 7 days, glucocorticoids strongly and persistently repressed osteocytic expression of the key PLR enzyme MMP13 in both trabecular and cortical bone of the mandible. Cathepsin K expression was significantly reduced only after 55 days of glucocorticoid treatment, at which point histological analysis revealed a glucocorticoid-dependent reduction in the lacunocanalicular surface area. In addition to reducing bone mass and suppressing PLR, glucocorticoids also reduced the stiffness of mandibular bone in flexural tests. Thus, osteocyte PLR in the neural crest-derived mandible is susceptible to glucocorticoids, just as it is in the mesodermally-derived femur, highlighting the need to further study PLR as a target of drugs, and radiation in mandibular osteonecrosis.

1. Introduction

Despite the common features of bone throughout the skeleton, bones retain several features that are anatomically distinct, including embryonic derivation, geometry, microarchitecture, and material properties. For example, the femur is mesodermally-derived and has robust cortical bone, whereas the mandible is derived of neural crest and is rich in trabecular bone. While such differences allow bones to meet their unique mechanical demands, different skeletal sites exhibit susceptibility to different clinical problems. Though considerable effort has focused on the restoration of bone mass in the femur or vertebra, many questions remain about the best therapies to treat craniofacial manifestations of skeletal disease such as osteonecrosis of the jaw following radiation therapy, a major unmet clinical need (Cha et al., 2017). Therefore, we seek to examine the role of osteocyte-mediated perilacunar/canalicular remodeling in the mandible.

Bone embedded osteocytes orchestrate bone remodeling by osteoblasts and osteoclasts, and also execute the remodeling of bone matrix directly through a process called perilacunar/canalicular remodeling (PLR). In PLR, osteocytes secrete acid and enzymes, including MMP-2, MMP-13, MMP-14, and cathepsin K (Tang et al., 2012; Inoue et al., 2006; Holmbeck et al., 2005), to resorb bone matrix in the perilacunar and pericanalicular microenvironments. PLR is activated during metabolic stress to increase circulatory levels of calcium and phosphate (JAMA, 1968; Bélanger et al., 1968; Marie and Glorieux, 1983; Zallone and Mueller, 1969; McGee-Lawrence et al., 2011), and is a fundamental process in bone homeostasis. Suppression of PLR, through ablation of PLR enzymes or in skeletal disease, causes severe degeneration of the lacunocanalicular network and compromises bone quality (Tang et al., 2012; Inoue et al., 2006; Holmbeck et al., 2005; Alliston, 2014; Dole

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et al., 2017; Fowler et al., 2017; Kogawa et al., 2013). Through mechanisms that remain unclear, PLR helps to maintain collagen organization and calibrates the mineralization of bone extracellular matrix. Consequently, disruption of the PLR leads to increased bone fragility (Tang et al., 2012; Dole et al., 2017).

PLR is tightly regulated by several factors, including parathyroid hormone, sclerostin, glucocorticoids, vitamin D, and TGFβ (Dole et al., 2017; Fowler et al., 2017; Kogawa et al., 2013; Qing et al., 2012; Rolvien et al., 2017). In addition, PLR is spatially regulated, such that it varies with anatomic location (Inoue et al., 2006). For example, Jauregui, et al., demonstrated that cochlear bone uniquely maintains bone quality and hearing independently of MMP-13-mediated PLR, which is critical for femoral bone quality (Jáuregui et al., 2016). Because of the mandible's unique derivation from the neural crest cells of the branchial arches rather than mesodermal origin, the question is raised as to whether PLR is differentially regulated in the mandible compared to mesodermally-derived long bones.

Among the many effects of glucocorticoids on bone, they are known to suppress PLR in trabecular and cortical bone of the femur, which likely plays a causal role in glucocorticoid-induced osteonecrosis of the femoral head (Fowler et al., 2017). In the mandible, glucocorticoids alter the biomechanical behavior of the bone as well as decrease overall cortical bone mass and strength (Bozzini et al., 2015; Kozai et al., 2009). However, the extent to which PLR in the mandible is sensitive to glucocorticoids has yet to be studied. Osteonecrosis of mandibular bone occurs in patients with autoimmune diseases, such as vasculitis, who are being treated with high dose steroids (Cowan et al., 1995). Although the extent to which glucocorticoids are responsible for this pathology is unclear, an understanding of the role of PLR in the mandible could improve strategies to prevent and treat osteonecrosis, particularly in this high risk population (Strojan et al., 2017; Chronopoulos et al., 2018). This is important because there are relatively limited options for surgical reconstruction of the necrotic mandible, which most commonly results as a late complication of external beam radiation (Strojan et al., 2017; Chronopoulos et al., 2018). Therefore, in this study, we investigated the extent to which glucocorticoids regulate PLR in the mandible, with the goal of advancing our understanding of osteocyte-mediated bone remodeling in mandibular disease.

2. Materials & methods

2.1. Murine studies

All animal procedures described herein were performed according to national ethical guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at University of California San Francisco. In an established model of glucocorticoid-excess, two-month-old male FVB mice were subcutaneously implanted with slow-release pellets containing placebo or prednisolone (2.8 mg/kg/day) (Innovative Research of America) and sacrificed at 7, 21, 35 and 55 days (n = 8, 8, 5, 6 per group, respectively) (Lane et al., 2006). Mandibles and femora were dissected for the following analyses.

2.2. Murine bone micro-computed tomography (μCT)

Hemimandible specimens were harvested and fixed overnight in 10% neutral buffered formalin and transferred to 70% ethanol for radiographic scanning. Radiologic analysis was performed using a desktop cone-beam micro-computed tomography scanner (μCT 40, Scanco Medical, Bruttisellen, Switzerland) and μCT valuation Software v6.0 (Scanco Medical). Specimens were scanned in 70% ethanol, at an energy of 109 kVp with a voxel size of 10 μm. Scans were reconstructed and three-dimensional digitized images were generated for each specimen (Fig. 1A).

Volumetric measurements were carried out following the selection of a standardized region of interest (ROI), which was comprised of the alveolar bone surrounding the roots of molars M1, M2 and M3 (Fig. 1B). The length of the ROI extended from the most mesial aspect of the M1 root to the most distal aspect of the M3 root. The width of the ROI extended from the most buccal aspect of any root of the molars to the most lingual aspect of any root. The height of the ROI extended from the inferior most aspect of any root to the alveolar bone crest (ABC) (Chen et al., 2015). A single blinded investigator drew the contour of the desired alveolar bone region so as to maximize the quantification of bone and minimize the inclusion of roots. The abovementioned landmarks defined the borders of the volumetric ROI that was analyzed in 2-D parasagittal images. Bone volume per total volume (BV/TV), trabecular thickness (TT), trabecular separation, trabecular number, Structural Model Index, and bone mineral density were then calculated from each specimen as described (Bouxsein et al., 2010).

2.3. Histology

For paraffin sectioning, dissected murine mandibles were fixed in 10% neutral buffered formalin and incubated in 10% di- and tetra-sodium EDTA for 20–25 days until fully decalcified, followed by serial ethanol dehydration and paraffin embedding. Paraffin sections (7 μm thick) in the sagittal plane of hemimandibles were generated using a microtome (Leica) for polarized light microscopy, Ploton silver stain and tartrate-resistant acid phosphatase (TRAP) staining (Dole et al., 2017; Fowler et al., 2017). In order to standardize evaluation, the trabecular bone was routinely evaluated in the area between molars M1 and M2, whereas cortical bone analysis was performed on the inferior border of the mandible.

Polarized light microscopy was performed as previously described (Jáuregui et al., 2016). Briefly, paraffin-embedded sections were stained in a saturated aqueous solution of picric acid and 0.1% Direct Red-80 (aka: Picrosirius Red) (Sigma-Aldrich). Slides were imaged using polarized filters, which were rotated to achieve the maximum birefringence. Images taken in these conditions were analyzed to quantify collagen fiber orientation.

Ploton silver stain was used to visualize the lacunocanalicular network (Kogawa et al., 2013). Briefly, paraffin-embedded sections were deparaffinized and rehydrated, then incubated in a solution of two-parts 50% silver nitrate and one-part 1% formic acid with 2% gelatin (Fisher Scientific) for 55 min. Stained slides were then washed in 5% sodium thiosulfate (Baker Chemicals) for 10 min and subsequently dehydrated, cleared, and mounted. Image J was used to threshold grayscale images for quantitative analysis of lacunocanalicular area, which was normalized to total bone area analyzed for each image.

For immunohistochemistry, slides were deparaffinized and hydrated prior to incubation in Innovex Unitrieve low temperature retrieval solution in a 60 °C water bath for 30 min. Endogenous peroxidase activity was quenched using 3% H_2O_2 for 10 min at room temperature. For all following steps, Innovex Universal Animal IHC kit was utilized. Background buster was applied for 60 min at room temperature. Slides were incubated with rabbit primary antibodies against MMP13 (Abcam #39012, diluted 1:50) and cathepsin K (Abcam #19027, diluted 1:75) in a humid chamber at 37 °C for 24 h. Subsequent incubations with anti-rabbit secondary antibody conjugated to strep avidin, and biotinylated horseradish peroxidase were both performed at room temperature for 10 min each. Fresh DAB solution was applied and incubated at room temperature for 5 min. Slides were mounted with Innovex Advantage Mounting medium. Negative controls were performed by substituting Innovex rabbit negative control sera in place of primary antibody. Quantification was performed with the help of Image J cell counter to determine the average percentage of MMP-13-positive or cathepsin K-positive osteocytes, relative to the total number of osteocytes in the visual field.

For TRAP staining, slides were deparaffinized and hydrated prior to incubation. TRAP solution was prepared according to the package