Assessment of MMP-2, MMP-3, And Osteocyte Apoptosis in The Lacunar-canalicular Network of Estrogen-deficient Rats

Divya Sharma¹, Andrew Moon¹, Damien M. Laudier¹, Stephen B. Doty², Mitchell B. Schaffler³, Susannah P. Fritton¹.
¹City College of New York, New York, NY, USA, ²Hospital for Special Surgery, New York, NY, USA.


Introduction: Postmenopausal bone loss is associated with reduced estrogen levels, increased bone remodeling, and increased osteocyte apoptosis. While it is established that estrogen deficiency reduces bone volume fraction, especially in cancellous bone, our recent studies using high-resolution microscopy demonstrate that estrogen deficiency also alters the submicron lacunar-canalicular porosity surrounding osteocytes in cortical and cancellous bone [1]. We found that the increase in effective canalicular size measured using a small molecular weight tracer in ovariectomized rats was due to nanostructural matrix-mineral changes at the osteocyte lacunar-canalicular surface [1]. Recent studies of lactating mice have also shown that osteocytes remodel their surroundings by enlarging lacunae and canaliculi [2]. Because alterations in the osteocyte microenvironment have the potential to alter cell viability and mechanotransduction, the purpose of this study was to evaluate osteocyte apoptosis as well as potential candidates of bone degradation in the osteocyte lacunar-canalicular network in estrogen-deficient rats. Two matrix metalloproteinases, MMP-2 and MMP-3, were chosen for evaluation because they can cleave substrates found in the osteocyte lacunar-canalicular microenvironment, and they have been shown to be produced in bone in the estrogen-deficient state [3,4]. Temporal assessment of osteocyte apoptosis, as measured by cleaved caspase-3, as well as MMP-2 and MMP-3 were quantified in the osteocyte lacunar-canalicular network using the rat ovariectomy model of postmenopausal osteoporosis.

Methods: Permission for this study was granted by the IACUC. Fifty-four female 20-week-old Sprague Dawley rats (Harlan) were divided into two groups, with one group undergoing ovariectomy (OVX, n=27) and the other group acting as sham-operated controls (SHAM, n=27). Nine rats from each group were sacrificed at three time points: 1, 2, and 6 weeks post-surgery. MMP-2 and MMP-3 presence in osteocytes was quantified for a subset of rats (OVX, n=6 and SHAM, n=6 per time point), and osteocyte apoptosis was measured in a separate subset (OVX, n=3 and SHAM, n=3 per time point). The analysis was focused on the proximal tibia, where changes in the osteocyte lacunar-canalicular network were previously observed in the rat OVX model [1]. The proximal tibiae were harvested and fixed in zinc-buffered formalin for 72 hours at 4º C. Samples were then decalcified in formic acid and embedded in paraffin for the osteocyte apoptosis (caspase) analysis or methacrylate for the MMP analysis. Cross sections were cut from the cortical tibial metaphysis.

To assess MMP and caspase presence using immunohistochemistry, sections were deparaffinized or deplasticized, rehydrated, and blocked. Specimens were incubated in a humidified chamber overnight with either MMP-2 (1:500), MMP-3 (1:50) (Abcam), or cleaved caspase-3 (1:1000) (Cell Signaling) primary antibody. Detection was performed using secondary antibody (HRP polymer) and developed
with DAB substrate chromogen system. Sections were counterstained and coverslipped. Optimal dilution for the primary antibody was determined using internal positive control tissues (growth plate or articular cartilage). Negative staining controls were established by applying rabbit serum without primary antibody.

The cortical metaphysis sections were divided into four sectors (apoptosis analysis) or eight sectors (MMP analysis). Within each sector, three 87.5 µm x 77.5 µm regions of interest were analyzed, and stained and non-stained osteocytes were counted under brightfield microscopy at 40x magnification. At least 200 osteocytes were assessed per rat for each analysis, and all analyses were blinded.

The percent cleaved caspase-3 positive osteocytes (% casp+ Ot) and the percent MMP-2 and MMP-3 positive osteocytes (% MMP2+ Ot and % MMP3+ Ot) were analyzed using two-way ANOVA with Bonferroni post-hoc tests for the two factors: treatment (OVX and SHAM) and time (1, 2, and 6 weeks post-surgery). The statistical analyses were performed using Prism 5 (GraphPad) with a significance level of p < 0.05.

**Results:** Ovariectomy caused an approximately 4-fold increase in overall osteocyte apoptosis within the tibial metaphyseal cortex at 1 and 2 weeks post-surgery, while at 6 weeks apoptosis was not significantly different in OVX compared to SHAM (Fig. 1). Significant changes were not observed in MMP-2 and MMP-3 presence in osteocytes in SHAM and OVX at any time point (Figs. 2 & 3). However, an increase in MMP-3 levels in both SHAM and OVX was observed 6 weeks post-surgery (Fig. 3).

**Discussion:** The increase in apoptotic osteocytes at 1 and 2 weeks post-OVX was similar to the temporal increases in osteocyte apoptosis reported in a mouse OVX model [5]. The precise mechanism by which osteocyte viability is altered is not known; however, the increased osteocyte apoptosis following estrogen loss may be a result of increased oxidative stress to the osteocytes [6]. It has also been postulated that alterations in interstitial fluid flow via changes of the osteocyte tethering connections to the canalicular wall may be related to osteocyte apoptosis [7]. Future studies assessing osteocyte apoptosis in the rat OVX model should include analysis of trabecular bone and osteoclast activity in the proximal tibia in order to elucidate bone resorption and spatial and temporal patterns of apoptosis that may exist in the cancellous compartment, which goes through a high remodeling phase following estrogen loss.

Because MMP-2 and MMP-3 levels were relatively unmodulated in the lacunar-canalicular network in the rat OVX model, it appears that these MMPs do not play a critical role in bone degradation around osteocytes after estrogen withdrawal. At 6 weeks post-surgery there was an increase in MMP-3 in both OVX and controls, which might be due to periosteocytic remodeling of non-collagenous proteins after the rat has reached its peak skeletal growth. Future work should test other MMPs, such as MMP-13 and MMP-14, to see whether they may be involved in the previously observed osteocyte lacunar-canalicular changes. MMP-13 has recently been shown to be necessary for lactation-induced osteocyte perilacunar remodeling [8], and MMP-14 has recently been shown to modulate mechanosensitivity in osteocytes [9].

**Significance:** The goal of this project is to better understand the cellular-level bone degradation process in postmenopausal osteoporosis, contributing knowledge that could aid in the design of clinical strategies to prevent and treat this disease.
Fig. 1. Osteocyte apoptosis (% caspase positive osteocytes, mean ± SD) at each time point. *p < 0.05.

Fig. 2. Percent osteocytes positive for MMP-2 (mean ± SD) at each time point.
Fig. 3 Percent osteocytes positive for MMP-3 (mean ± SD) at each time point. *p < 0.05.