Osteocytes As MechanoSensors in the inhibition of Bone resorption due to mechanical loading

**INTRODUCTION:** Bone has the ability to adjust its structure to meet its mechanical environment. Osteocytes are believed to be responsible for detecting and responding to mechanical loading and initiating bone remodeling process [1, 2]. However, how osteocytes regulate bone remodeling is not well understood. Recent studies have shown that osteocytes support osteoclast formation and express RANKL on their cell surface and cell junction membrane proteins both in vitro and in vivo [3]. In this study, we examined the osteocytes’ inhibitory effect on osteoclast formation under mechanical loading. Osteocytes subjected to oscillatory fluid flow (OFF) exhibited a decreased RANKL/OPG ratio at the mRNA level, and inhibited osteoclastogenesis when co-cultured with monocyte macrophage osteoclast precursors. These osteoclast precursors, treated w/ or w/o conditioned media (CM) from osteocytes, did not develop osteoclasts when no soluble RANKL (sRANKL) and M-CSF were added (data not shown). However, adding CM from osteocytes to either osteoclast precursors cultures with physiologic circulating levels of sRANKL and M-CSF [4], or to a co-culture of osteoclasts/stromal cells with osteoclast precursors, decreased the osteoclast formation. Moreover, the CM from osteocytes exposed to OFF further enhanced the inhibitory effect in the above experimental paradigms.

**METHODS:** Cell culture: MLO-Y4 cells (gift from Dr. Lynda Bonewald, University of Missouri-Kansas City) were used to model osteocytes. They were cultured on type 1 rat tail collagen coated plates in αMEM (5% FBS, 5% CS, 1% PS). RAW264.7 cells were used to model monocyte macrophage osteoclast precursors, and they were cultured in DMEM (10% FBS and 1% PS). ST2 cells were used to model stromal cells, and they were cultured in αMEM (10% FBS and 1% PS). Osteoclastogenesis models: Three osteoclastogenesis models were used in this study: the culture of MLO-Y4 and RAW264.7 cells, co-culture of ST2 and RAW264.7 cells, and RAW264.7 cells w/ sRANKL (0.1 ng/ml) and M-CSF (1 ng/ml). Note, the amount of sRANKL and M-CSF used in this study was at circulating level [4], which is significantly lower than the level normally used for osteoclastogenesis studies in the literature. In the first two co-culture models, MLO-Y4 or ST2 cells were seeded at 500 cells/cm², RAW264.7 cells were added at 2500 cells/cm² at day 2; cells were then co-cultured in DMEM containing 10% FBS for 7 days (for ST2 cells, ViD) was added at 10 mM to the medium). Medium was replaced every 2 or 3 days. At the end of co-culture, cells were fixed and stained for TRAP. Osteoclasts were identified as TRAP positive cells containing three or more nuclei (TRAP+ MNCs). The effect of soluble factors released by osteocytes on osteoclastogenesis was studied using CM from MLO-Y4. After the flow experiment, slides were transferred from flow chamber to plates, and the cells were allowed to sit for one day. Conditioned media was then collected and added to the desired culture system w/ or w/o M-CSF and sRANKL. Oscillatory Fluid Flow (OFF): MLO-Y4 cells were seeded on type 1 rat tail collagen coated slides for 2 days prior to flow exposure. Cells were exposed to OFF at peak shear stresses of 10 dynes/cm² at 1 Hz for 2 hrs. The controls for the flow experiments were the cells cultured on slides placed in flow chambers but exposed to no flow (NF). RT-PCR: Total RNA was extracted from slides after flow using Tri Reagent. cDNA was reverse transcribed from total RNA and then amplified using Taqman PCR Master Mix, 20X primer and probes. Amplifications were reverse transcribed from total RNA and then amplified using Taqman PCR Master Mix, 20X primer and probes. Amplifications were performed using the ABI Prism 7900HT Sequence Detection system. Statistics analysis: For all two sample comparisons, student t test was used. For all multiple comparisons, post-hoc ANOVA using Bonferroni test was used. p < 0.05 is considered significant. Data was reported as mean ± SE.

**RESULTS:** OFF decreased RANKL/OPG ratio at the mRNA level: Both RANKL and OPG were upregulated in MLO-Y4 after 2 hr OFF exposure, but the ratio of RANKL/OPG was decreased by 30% (Fig. 1, left).

OFF inhibited osteoclast formation: MLO-Y4 cells exposed to 2 hr OFF induced 35% fewer osteoclasts when placed in co-culture with RAW264.7 cells that did not experience flow (Fig. 1, right). CM from MLO-Y4 did not support osteoclastogenesis (data not shown). However, CM from MLO-Y4 exposed to 2 hr OFF inhibited osteoclast formation by direct contact of RAW264.7 cells with MLO-Y4 (26%) or ST2 (31%) cells (Fig. 2, left). Furthermore, CM from MLO-Y4 w/o flow exposure decreased the osteoclast formation by 9% in RAW264.7 cells w/ circulating levels of sRANKL and M-CSF. This inhibition was further enhanced (11.4%) when the CM from MLO-Y4 exposed to 2 hr OFF was added (Fig. 2, right).

**DISCUSSION:** In this project we have demonstrated that mechanical stimulation experienced by osteocytes has an anti-resorptive effect on the RANKL/OPG signaling axis. We found that mechanical stimulation of osteocytes reduced osteoclast formation known to occur with cell-cell contact. We confirmed that signaling via soluble factors is not responsible for osteocyte mediated osteoclast formation. Interestingly, however, we were able to demonstrate that these soluble signals released by osteocytes are able to affect osteoclast formation without cell-cell contact through three distinct mechanisms: 1) inhibition of osteoclastogenesis mediated by cell-cell contact with stromal/pre-osteoblastic cells, 2) inhibition of osteoclastogenesis mediated by cell-cell contact with osteocytes, and 3) inhibition of baseline osteoclastogenesis due to physiologic circulating levels of sRANKL and M-CSF. Our results suggest an interesting novel mechanism whereby osteocytes can regulate osteoclast formation via soluble signals and that this mechanism plays an important in the anti-resorptive effects of mechanical loading.


**ACKNOWLEDGEMENT:** This work was supported by NIH Grant AR 45899, US Army Medical Research Award DAMD 17-98-1-8509, NASA NAG2-1601, VA RR&D Center.