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Osteocytes as Mechanosensors in the Inhibition of Bone Resorption Due to Mechanical Loading

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Abstract

Bone has the ability to adjust its structure to meet its mechanical environment. The prevailing view of bone mechanobiology is that osteocytes are responsible for detecting and responding to mechanical loading and initiating the bone adaptation process. However, how osteocytes signal effector cells and initiate bone turnover is not well understood. Recent *in vitro* studies have shown that osteocytes support osteoclast formation and activation when co-cultured with osteoclast precursors. In this study, we examined the osteocytes' role in the mechanical regulation of osteoclast formation and activation.

We demonstrated here that 1) Mechanical stimulation of MLO-Y4 osteocyte-like cells decreases their osteoclastogenic-support potential when co-cultured with RAW264.7 monocyte osteoclast precursors, 2) Soluble factors released by these mechanically stimulated MLO-Y4 cells inhibit osteoclastogenesis induced by ST2 bone marrow stromal cells or MLO-Y4 cells, and 3) Soluble RANKL and OPG were released by MLO-Y4 cells, and the expressions of both were found to be mechanically regulated.

Our data suggests that mechanical loading decreases the osteocyte's potential to induce osteoclast formation by direct cell-cell contact. However, it is not clear that osteocytes *in vivo* are able to form contacts with osteoclast precursors. Our data also demonstrates that mechanically stimulated osteocytes release soluble factors that can inhibit osteoclastogenesis induced by other supporting

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cells including bone marrow stromal cells. In summary, we conclude that osteocytes may function as mechanotransducers by regulating local osteoclastogenesis via soluble signals.

Keywords

Osteocyte; osteoclast; RANKL; OPG; mechanotransduction

Introduction

It is well known that bone can adjust its structure to become better suited to withstand the mechanical demands it experiences. Physical loading and routine activities have been shown to inhibit bone resorption that would otherwise occur with disuse (3,8,12,26). However, the cellular mechanism underlying this phenomenon remains largely unknown. The focus of this investigation was to determine the mechanisms by which osteocytes might transduce and regulate bone resorption and the anti-resorptive effects of loading.

Osteocytes inhabit a fluid filled network made up of widely spaced lacunae and are interconnected via cellular processes contained within thin channels known as canaliculi. These fluid-filled lacunae and canaliculi also contain a proteoglycan-rich extracellular matrix which affects the diffusion of soluble factors released by osteocytes. Two key features of osteocytes as mechanosensors are their ability to detect mechanical stimuli and to send signals to other effector cells that regulate bone formation and resorption (5,6,56,58,59). Dynamic fluid flow is one of the mechanical stimuli that osteocytes experience *in vivo* with habitual loading(24, 25,54). Previous studies have established that this loading-induced dynamic fluid flow is a potent physical signal in the regulation of bone cell metabolism (7,16,37,57), yet it is unclear what role it might play in osteocyte mediated regulation of bone resorption.

The effector cells of bone resorption are osteoclasts, which are transient multinucleated cells that arise from haemopoietic cells of the monocyte/macrophage lineage. Osteoclast formation *in vivo* is thought to be induced by direct cell-cell contact of pre-osteoblastic/stromal cells with monocyte/macrophage osteoclast precursors (4,38). Two key molecules have been found to mediate this interaction: receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) (also known as TRANCE, OPGL or ODF) and osteoprotegerin (OPG) (also know as OCIF). RANKL stimulates osteoclast precursors to commit to the osteoclastic phenotype by binding to its receptor (RANK) on the surface of osteoclast precursors. RANKL exists in two forms, a soluble form (sRANKL) and a membrane bound form thought to be responsible for initiating osteoclast formation (4,52). sRANKL is found in the circulatory system of both animals and human (35,46,53,61). It is thought to play a role in immune response and bone repair (27,34, 39,46) and recently has been shown to decrease with endurance physical activity (61). A recent study by Hikita *et al*. (11) indicated that soluble RANKL can be secreted by primary osteoblasts. However, membrane-bound RANKL was found to be much more efficient in terms of inducing osteoclast formation than soluble RANKL (11). OPG is a decoy receptor that can bind to RANKL and inhibits its binding with RANK. OPG binding to RANKL not only blocks osteoclastogenesis but also decreases the survival of pre-existing osteoclasts. The bone resorption rate is thus affected by the balance of RANKL and OPG (4).

Osteoclastogenesis has been shown to be regulated by mechanical loading both *in vitro* and *in vivo* (17,19,21,40-43). Rubin and colleagues (40-43) have shown that dynamic mechanical strain can decrease osteoclast formation by about 50% in primary marrow cultures, and this is mediated through a decrease in RANKL and an increase in eNOS-generated NO in bone marrow stromal cells. OPG expression at both the gene and the protein level has also been shown to be regulated by mechanical stimulation (21,45). A recent study by Kim *et al.* (21)

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reported that physiological levels of loading-induced fluid flow decreased osteoclast formation in a co-culture system of marrow stromal cells and osteoclast precursors by decreasing the RANKL/OPG mRNA ratio. Taken together, these studies suggest that osteoclastogenesis in the marrow stroma can be mechanically regulated at the cellular level via the RANKL-OPG-RANK signaling system. However, it is unclear what role osteocytes might play in the mechanical regulation of osteoclastogenesis.

Osteocytes have been shown to support osteoclastogenesis when co-cultured with osteoclast precursors (60). Interestingly however, this induction was found to require cell-cell contact since the conditioned media of osteocytes was not able to support osteoclast formation. It is possible that osteocytes near the bone surface or in active resorption pits sense mechanical loading and regulate osteoclast formation via their expression membrane-bound RANKL. However, the opportunity for osteocytes to come in direct contact with monocytic osteoclast precursors is likely to be limited. Thus, another important question is whether soluble factors released by osteocytes might regulate osteoclast formation supported by stromal cells.

In this study we address the potential for mechanical stimulation to regulate the ability of osteocytes to support osteoclastogenesis by direct cell-cell contact with osteoclast precursors and whether the RANKL/OPG signaling axis is the relevant mechanism. Additionally we show that mechanically stimulated osteocytes secrete soluble signals that are able to inhibit the osteoclast formation induced by contact with other cells including bone marrow stromal cells.

Materials and Methods

Cell Culture

Three cell lines were used in this study. MLO-Y4, kindly provided by Dr. Lynda Bonewald (University of Missouri-Kansas City, Kansas City, MO), is an immortalized cell line that has properties very similar to primary osteocytes in terms of morphology and several important molecular osteocyte markers (20). RAW264.7 (ATCC) is a mouse monocyte/macrophage cell line which can differentiate into multinucleated cells with an osteoclastic phenotype that possess the ability to resorb calcified substrates. ST2 (Riken, Japan) is a murine bone marrow stromal cell line. These cells can differentiate into osteoblast-like cells given appropriate stimulation and can support osteoclast formation when co-cultured with osteoclast precursors.

MLO-Y4 cells were cultured on type I Rat tail collagen (BD Laboratory) coated plates in αMEM, (GIBCO™) supplemented with 5% fetal bovine serum (FBS), 5% calf serum (CS), and 1% penicillin and streptomycin (PS). RAW264.7 cells were cultured in DMEM (GIBCO™) supplemented with 10% FBS and 1% PS. ST2 cells were maintained in αMEM supplemented with 10% FBS and 1% PS. All cell lines were maintained at 37°C and 5% CO_2 in a humidified incubator. For flow experiments, MLO-Y4 cells were cultured on type I rat tail collagen-coated glass slides (75mm × 38mm × 1 mm) 48 hours prior fluid flow exposure at 200,000 cells/slide to ensure the 80–90% confluence at the time of experiment.

Multiple osteoclast formation strategies were employed in this project. Co-culture of MLO-Y4 and RAW264.7 cells was used to explore the role of osteocytes in the regulation of osteoclastogenesis by direct cell-cell contact. MLO-Y4 cells were seeded at 500 cells/cm^2 in collagen-coated 24-well plates (day 0). RAW264.7 cells were added at 2500 cells/cm^2 at day 2 and then co-cultured in DMEM containing 10% FBS for 7 days. Pre-osteoblast/stromal cell induced osteoclast formation was similarly performed as described above except that ST2 cells were substituted for MLO-Y4 cells. For these experiments media was supplemented with 10nM 1α,25-dihydroxyvitamin D3 (Fluka) for RANKL expression and the plates were not collagen coated.

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In all osteoclastogenesis systems described above, medium was replaced every 2 or 3 days. At day 7 starting from RAW264.7 cell plating, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (385A, SIGMA) as instructed in the product manual. Osteoclasts were identified as TRAP positive cells containing three or more nuclei (TRAP+ MNCs). These cells were counted using a light microscope with 20× objective by 2 blinded investigators. Each sample was counted three times, and the average value of these repeats was reported as the TRAP+ MNCs number for each sample.

To assess osteoclast activity, cell culture was performed as described above for each osteoclastogenesis system except that the substrates were dentine discs (Osteosite™) in 96-well plates. The cells were cultured for 14 days (starting from RAW264.7 cell plating). At day 15 cells were removed from dentine discs using a toothbrush. The dentin discs were then stained with 1% toluidine blue for 2 minutes, rinsed in distilled water and air dried. The existence of osteoclast-resorbed lacunae were observed and verified under a light microscope.

The effect of soluble factors released by osteocytes on osteoclastogenesis was studied using conditioned medium from MLO-Y4 cells. After flow exposure, cell seeded glass slides were transferred from flow chambers to sterile petri dishes with 15 mL fresh media added. At 24 hours post flow, conditioned medium samples were collected from these dishes and added immediately to the co-culture of RAW264.7 and MLO-Y4 or ST2 cells to replace 50% of the original culture medium. The collected conditioned medium was never frozen or allowed to age in any way. This process is repeated every subsequent day until day 7 (starting from RAW 264.7 cell plating) when cultures were stained for TRAP activity.

Oscillatory Fluid Flow

Our lab has previously shown that oscillatory fluid flow is a potent regulator of bone cell metabolism (2,21,30). A 2 hour exposure to oscillatory fluid flow was selected as the mechanical stimuli in this study based on a previous experiment where this exposure period was found to maximally impact the RANKL/OPG ratio (21).

A previously established flow system was used to apply oscillatory fluid flow in this study (16,31). In brief, flow was driven by a Hamilton glass syringe, which was mounted on and driven by an electromechanical loading device (EnduraTEc). An oscillatory fluid flow pattern with a peak flow rate of 27 mL/min was generated which yields a peak sinusoidal wall shear stress of 1 Pa at 1 Hz in flow chambers. For all flow experiments, MLO-Y4 cells were seeded on collagen-coated slides for 2 days, and then exposed to the oscillatory fluid flow pattern described above for 2 hours. Cells cultured on slides, placed in flow chambers, but not exposed to flow were the experimental controls. All flow chambers were placed in a CO_2 incubator for the entire duration of the flow experiment.

Quantitative real time RT-PCR analysis of steady-state mRNA levels

Immediately following the completion of the flow experiment, cell-seeded glass slides were transferred from flow chambers to 100 mm sterile petri dishes. Total RNA was extracted from cells using Tri-Reagent (SIGMA). Extracted RNA was used for cDNA synthesis by reverse transcriptase using GeneAmp RNA PCR Core Kit (Applied Biosystems). The cDNA samples were subjected to PCR analysis using Taqman PCR Master Mix and 20× primer and probes (Applied Biosystems). Amplifications were then performed using the ABI Prism 7900HT Sequence Detection system. The expression of the gene of interest and the housekeeping gene (18S) were simultaneously determined in the same sample. For each sample, mRNA levels for each gene were normalized to 18s rRNA levels.

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Protein Quantification

Immediately following the completion of flow exposure, cell seeded slides were transferred from flow chambers to sterile petri dishes, with 15 mL of fresh media added, and returned to the incubator. Media samples were collected at 2 hours, 24 hours, and 48 hours post flow. Supernatant levels of RANKL and OPG were measured using Quantikine Mouse RANKL Immunoassay and Quantikine Mouse OPG Immunoassay, respectively, from R&D systems. Total protein assay was performed using BCA™ Protein Assay Kit from Pierce.

Statistical analysis

For two sample comparisons, Students *t* test was used. To compare observations from more than two groups, ANOVA was used. A significance level of 0.05 was employed for all statistical analyses. Data were reported as mean +/− SE.

Results

Osteocytes support osteoclast formation and activation when direct cell-cell contact with osteoclast precursors was allowed

It has been shown that osteocytes can support osteoclast formation and activation using co-culture systems of MLO-Y4 cells and spleen/marrow cells (60). As expected MLO-Y4 cells can also support osteoclast formation from RAW264.7 cells at multiple cell densities and ratios. We found that the co-culture of MLO-Y4 cells at 1000 cells/well and RAW264.7 cells at 5000 cells/well resulted in maximal osteoclast formation (data not shown). Figure 1 shows that tartrate-resistant acid phosphatase (TRAP) positive cells containing three or more nuclei (TRAP+ MNCs) were formed in this co-culture system at day 9 and pits were formed on dentine discs after 14 days of co-culture.

Oscillatory fluid flow decreases MLO-Y4 cells osteoclastogenic-support potential

To test whether oscillatory fluid flow exposure would affect the rate of osteoclast formation in the system described above, we seeded the MLO-Y4 cells on slides at a density of 2×10^5/slide for 48 hours and then exposed them to oscillatory fluid flow for 2 hours. Next, RAW264.7 cells were deposited on the slides; cells were then co-cultured and TRAP staining was performed at day 9. We found that 2 hours of flow exposure decreased the TRAP+ MNCs formed in the co-culture by 38% ($p = 0.002$, experiment was repeated 4 times) (Figure 2). Note, to ensure that the observed decrease in osteoclast formation is not due to any potential loss of MLO-Y4 cells dislodged by the fluid flow shear stress, BCA total protein assay was conducted on lysed MLO-Y4 cells before and after flow. We found no changes in total protein level due to flow (data not reported).

Oscillatory fluid flow decreases RANKL/OPG ratio at the mRNA level in MLO-Y4 cells

To investigate the mechanism behind the down-regulation of osteoclastogenesis by oscillatory fluid flow on MLO-Y4 cells, we measured the RANKL/OPG ratio at the mRNA level in MLO-Y4 cells with or without flow exposure. MLO-Y4 cells were cultured on collagen-coated glass slides for 2 days and exposed to oscillatory fluid flow for 2 hours. Total RNA was collected at 0 hour, 24 hours, and 48 hours after flow exposure. Quantitative real time RT-PCR analysis was performed on RANKL, OPG and 18s rRNA. At 0 hour post flow, cells subjected to oscillatory fluid flow had significantly higher RANKL (107% increase, $p=0.037$) and OPG (164% increase, $p=0.017$) mRNA levels compared to the control groups (Figure 3). As a result, 2 hours oscillatory fluid flow exposure caused a decrease in RANKL/OPG mRNA ratio by 29% ($p=0.028$). However, at 24 hours and 48 hours post flow, the RANKL/OPG mRNA ratio was found to return to the pre-flow baseline level (data not shown).

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Osteocytes that are exposed to oscillatory fluid flow release soluble signals that inhibit osteoclast formation

We next investigated the potential role in osteoclastogenesis of soluble signals released by MLO-Y4 cells. No TRAP+ MNCs were observed when RAW264.7 cells were exposed to MLO-Y4 conditioned media for 7 days (data not shown). This indicates that soluble factors released by osteocytes do not induce osteoclastogenesis directly as expected given the results of Zhao *et al*. (60). However, it is not known whether the soluble factors released by mechanically challenged MLO-Y4 cells can affect osteoclastogenesis supported by contact with another cell type.

To determine whether the soluble factors released by mechanically stimulated MLO-Y4 cells can regulate osteoclastogenesis induced by other cells, at 24 hours post flow freshly collected conditioned medium from MLO-Y4 cells with or without exposure to oscillatory fluid flow was added to two systems that are known to result in osteoclast formation: (1) co-culture of RAW264.7 cells with ST2 cells, or (2) co-culture of RAW264.7 cells MLO-Y4 cells. For each group, the experiment is repeated 4 times (n=4). We observed a decrease of osteoclast numbers formed in both systems: 26% (p=0.0005) and 31% (p=0.006) (Figure 4), respectively.

Oscillatory fluid flow affects RANKL and OPG protein release by MLO-Y4 cells

MLO-Y4 cells were prepared as described previously. After 2 hours oscillatory fluid flow exposure, cell seeded slides were transferred from flow chambers to sterile petri dishes, and incubated for 2 days. Conditioned medium samples were collected from the culture dishes at multiple time points (2 hours, 24 hours, and 48 hours post flow) and ELISA was performed to quantify supernatant RANKL and OPG levels.

The two hours oscillatory fluid flow decreased the production of sRANKL at both 2 hours (10%, p=0.049) and 24 hours (59%, p=0.00003) post flow compared to no flow controls (Figure 5A). We next measured the supernatant OPG level in the conditioned medium from MLO-Y4 cells with or without 2 hours oscillatory fluid flow exposure (Figure 5B). At 2 hours post flow, OPG was significantly increased by 60% in cells exposed to 2 hour oscillatory fluid flow (p=0.0297). At 24 hours post flow, the increase of OPG is 77% greater in the flow group, however, the difference between the flow group and the no flow control group is not statistically significant (p= 0.068). Power analysis is conducted and the possibility of type II error is less than 0.6%.

Discussion

It has been shown that mechanical loading has the ability to suppress bone resorption (3,8, 12,26). However, the cellular mechanism that underlies this anti-resorptive effect is not clear. Osteocytes are the most abundant cell type in bone. Their location, embedded in the mineralized bone tissue, and demonstrated mechanosensitivity suggest they function as cellular mechanotransducers (5,23). Furthermore, recent studies have shown that osteocytes can support osteoclast formation and activation by direct cell-cell contact with osteoclast precursors (10,60). Thus, the purpose of this study was to determine if and by what mechanism osteocytes might regulate osteoclast formation due to mechanical loading.

The first experiment in this study was to determine if mechanical stimulation of osteocytes might affect their potential to induce osteoclast formation by direct cell-cell contact. Previously Zhao *et al.* (60) demonstrated that MLO-Y4 osteocytes can induce osteoclastogenesis when co-cultured with osteocyte precursors. We extended this finding by showing that mechanical stimulation has the effect of diminishing this induction. Our results showed that 2 hours of flow exposure inhibited osteoclast induction by MLO-Y4 cells by 38% (Figure 2).

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One concern with the concept of osteocyte mediated regulation of osteoclast formation is that osteocytes have limited physical contact with the hemopoietic osteoclast precursors *in vivo* - osteocytes are deeply embedded in bone matrix and do not appear to contact bone marrow except perhaps the few osteocytes adjacent to a bone surface (18). Given the fact that osteoclast formation *in vivo* is thought to require cell-cell contact between monocyte osteoclast precursors and supporting cells (e.g. pre-osteoblasts or bone marrow stromal cells), regulation of osteoclastogenesis directly via physical contacts between osteocytes and osteoclast precursors would be unlikely except on the resorption surface where osteocyte processes might be exposed. We therefore conducted an experiment to determine if mechanically stimulated osteocytes might release diffusible factors capable of regulating osteoclastogenesis at a distance. We exposed co-cultures of ST2 stromal cells and RAW264.7 monocytes to the conditioned media of osteocytes exposed to fluid flow. We observed a 31% decrease in osteoclast formation relative to the conditioned media from no flow controls. This finding is consistent with osteocytes acting as mechanosensors and mediating stromal cell induced osteoclastogenesis via diffusible factors. A similar result was obtained for osteocyte induced osteoclastogenesis (26% decrease). Thus, our data suggests that mechanically challenged osteocytes may affect the osteoclast-inducing capacity of supporting cells (stromal cells, osteoblasts, or osteocytes) via soluble factors resulting in changes in the supporting cells' local chemical environment. Note that the fluid flow to which osteocytes were exposed in this study is 2 hours. Several others studies (1,16,50,51,57) have shown that mechanical stimuli at this duration can induce many changes in markers of bone formation/resorption in bone cells. We expect that with longer flow exposure times, the bone resorption inhibition effect from mechanical loading might be further enhanced. The CM used in this study was from MLO-Y4 cells seeded on glass slides immersed in 15 ml medium to ensure that the cells remained covered during the subsequent 24 hour post flow incubation. Due to this dilution the concentration of our CM (ratio between cell numbers and medium volume) is relatively low compared to other studies of this type. Nevertheless, we observed an inhibitory effect of the CM on the osteoclastogenesis, suggesting that with higher concentration of this CM, the inhibition effect would be greater. Taken together these observations suggest that the mechanical regulation of bone resorption may occur via osteocyte regulation at a distance away from the actual site of osteoclastogenesis potentiated by other cell types. Furthermore, it is also possible that the soluble factors released by osteocytes might affect the osteoclast-support capacity of osteoblastic cells.

To explore the mechanism by which the anti-osteoclastogenic effects of osteocytes occur, we examined the key signaling molecules that regulate osteoclastogenesis, RANKL and OPG. Consistent with a previous study (60), we observed that osteocytes express RANKL and OPG at both the mRNA and protein levels. The RANKL/OPG mRNA ratio decreased immediately after flow. However, this decrease is lost with time and recovered to pre-flow baseline level at 24 hours and 48 hours. This result is consistent with our previous study on ST2 cells (21). It appears that the effect of mechanical loading on RANKL/OPG mRNA ratio in bone cells occurs immediately and when the stimulus is removed the signaling system is reset. In vivo stimulation would be continuous and although our data do not address this question directly, we would anticipate that this resetting would not occur and the decrease in RANKL/OPG mRNA ratio would be maintained.

OPG and sRANKL protein released by MLO-Y4 cells was then measured at 2 hour and 24 hour post flow. Two hours of flow exposure dramatically increased OPG message and protein (Figure 3 and Figure 5), suggesting that the inhibitory effect of flow might be due to an upregulation of OPG. However, at 24 hour post flow, OPG protein level is not statistically significant different (p=0.068) from the non-flow group. We speculate that in addition to OPG, other soluble factors such as TGF-beta and VEGF may also contribute to the decrease in osteoclastogenesis induced by CM from MLO-Y4 cells. Indeed, there is some evidence in the

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literature to support this view. For example, studies have shown that mechanical stimulation of osteocytes can upregulate TFG-beta and that osteocytes inhibit osteoclastic bone resorption through TGF-beta (10). Furthermore, it has been found that fluid shear can increase TGF-beta in osteoblastic cells (32,44). VEGF has been found to be increased after MLO-Y4 cells are exposed to fluid flow (49), and VEGF can increase osteoclastogenesis *in vitro* (47). Taken together with our findings, these results suggest that the inhibitory effect of MLO-Y4 cell CM on induced osteoclastogenesis involves one or more signaling molecules and may or may not involve OPG.

In non-osteocyte cell types RANKL mRNA has been reported to be downregulated with mechanical stimulation (21,41-43). Paradoxically, we observed an upregulation of RANKL mRNA with stimulation in osteocytes, suggesting RANKL mechanoregulation at gene level in osteocytes might be different from other cell types. Furthermore, we observed a decrease in sRANKL protein under the same flow conditions. The mechanism responsible for this discrepancy is not clear, however, it is likely to involve the recently uncovered and only partially understood complex RANKL regulation mechanism (13,48). Three isoforms of RANKL have been reported to occur as alternative splice variants (denoted RANKL1/2/3). Our PCR primers were designed to detect exon 526, common to all three isoforms. i*n vivo* sRANKL protein can be the translational product of RANKL3 mRNA, which only contains an extracellular domain (14). Thus, flow might increase the RANKL1 and RANKL2 expression but decrease RANKL3 expression so that the message level of RANKL is increased but the sRANKL protein as the translational product of RANKL3 is decreased. Alternatively sRANKL may be formed from the enzymatic cleavage of the extracellular domain of a full length RANKL (15,29,34,55). And the flow induced decrease of sRANKL may be the result of the flow exerted posttranscriptional or enzymatic effect on MLO-Y4 cells.

RANKL protein has been reported in bone cells *in vivo* (36) and *in vitro* (60). However, data on whether bone cells release soluble RANKL are difficult to interpret. Kusumi *et al*. (28) reported that normal human osteoblasts release sRANKL and this release was downregulated dramatically by applying continuous tensile strain to the cells. Ikeda et al. (14) found RANKL3 mRNA in ST2 and MC3T3-E1 cells. Nevertheless, many studies suggest bone cells either do not release or release very limited levels of soluble RANKL (9,22,33,45,60). In our study, we observed that MLO-Y4 cells released sRANKL into the media, and this was downregulated with mechanical stimulation. However, this released sRANKL was not able to directly induce osteoclast formation. Similarly, previous experiments showed that conditioned media from osteoblasts/stromal cells was not able to promote osteoclast formation. Thus, our findings suggest that RANKL dynamics in osteocytes is consistent with that reported for other types of bone cells, and the levels of sRANKL produced by bone cells are not able to induce osteoclastogenesis.

The existence of the pericellular matrix in the space surrounding osteocytes could potentially affect the diffusion of the soluble factors released by osteocytes including sRANKL and OPG. The molecular weight of sRANKL and OPG have been reported as 24kD and 60kD respectively. The tracer horseradish peroxidase (44kD, 6nm) has been found to diffuse through the osteocyte pericellular matrix (7) suggesting that indeed sRANKL and OPG are able to move through the lacunar canalicular system.

Although not the primary focus of this investigation, this work has certain implications for potential mechanisms whereby resorbing osteoclasts are targeted to sites where bone remodeling is required. We found that conditioned media from mechanically loaded osteocytes was able to inhibit osteoclast formation induced by cell-cell contact between ST2 cells and RAW264.7 cells. Thus, the physiologic condition (i.e. bone is regularly being mechanical loaded) may be a homeostatic equilibrium between baseline sRANKL and anti-resorptive

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factors locally secreted by osteocytes. In the absence of mechanically stimulated osteocytes the balance would locally favor osteoclast formation and activation. This would provide a compelling mechanism for the removal and replacement of dead osteocytes. In addition, our study is the first to demonstrate that osteocytes release diffusible chemical signals that regulate the resorptive potential in their local surroundings in response to changes in the physical signals they experience. Specifically, our results suggest that osteocytes under normal loading create a local chemical environment that is not osteoclastogenic. However, osteocytes that are not exposed to the appropriate physical signals shift the balance of secreted factors to favor resorption. Thus, in local regions of low flow (e.g., local areas of damaged bone tissue), osteocytes would enter a state of disuse and signal increased osteoclast formation. Furthermore, the changes in the local chemical environment surrounding the osteocyte in the absence of loading might also serve to attract and guide active osteoclasts to replace regions of damage and disuse.

In summary, we have demonstrated that mechanical stimulation may regulate osteoclast formation via osteocytes. Oscillatory fluid flow has been show to decrease osteoclast formation induced by cell-cell contact between osteocytes and osteoclast precursors. Furthermore, our results show that the osteoclast support capacity of non-osteocytes is modulated by soluble factors released by mechanically challenged osteocytes. This finding supports a novel osteoclast regulation mechanism. Specifically, we suggest that mechanically challenged osteocytes can affect the local chemical environment of marrow stromal and pre-osteoblastic cells via secreted signals that alter their ability to induce osteoclast formation. Such a mechanism would allow osteocytes to function as the local mechanotransducers to sense mechanical loading in mineralized tissue and communicate this information beyond the bone surface.

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References

1. Allen FD, Hung CT, Pollack SR, Brighton CT. Serum modulates the intracellular calcium response of primary cultured bone cells to shear flow. J Biomech 2000;33:1585–91. [PubMed: 11006382]
2. Batra NN, Li YJ, Yellowley CE, You L, Jacobs CR. Effects of short term recovery periods on fluid induced calcium oscillations in osteoblastic cells. J Biomech. 2004
3. Boyde A. The real response of bone to exercise. J Anat 2003;203:173–89. [PubMed: 12924818]
4. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003;423:337–42. [PubMed: 12748652]
5. Burger EH, Klein-Nulend J. Mechanotransduction in bone--role of the lacunocanalicular network. Faseb J 1999;13:S101–12. [PubMed: 10352151]
6. Cowin SC, Moss-Salentijn L, Moss ML. Candidates for the mechanosensory system in bone. J Biomech Eng 1991;113:191–7. [PubMed: 1875693]
7. Donahue SW, Jacobs CR, Donahue HJ. Flow-induced calcium oscillations in rat osteoblasts are age, loading frequency, and shear stress dependent. Am J Physiol Cell Physiol 2001;281:C1635–41. [PubMed: 11600427]
8. Duncan RL, Turner CH. Mechanotransduction and the functional response of bone to mechanical strain. Calcif Tissue Int 1995;57:344–58. [PubMed: 8564797]
9. Granchi D, Cenni E, Savarino L, Ciapetti G, Forbicini G, Vancini M, Maini C, Baldini N, Giunti A. Bone cement extracts modulate the osteoprotegerin/osteoprotegerin-ligand expression in MG63 osteoblast-like cells. Biomaterials 2002;23:2359–65. [PubMed: 12013183]

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10. Heino TJ, Hentunen TA, Vaananen HK. Osteocytes inhibit osteoclastic bone resorption through transforming growth factor-beta: enhancement by estrogen. J Cell Biochem 2002;85:185–97. [PubMed: 11891862]
11. Hikita A, Yana I, Wakeyama H, Nakamura M, Kadono Y, Oshima Y, Nakamura K, Seiki M, Tanaka S. Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappa B ligand. Journal of Biological Chemistry 2006;281:36846–36855. [PubMed: 17018528]
12. Hillam RA, Skerry TM. Inhibition of bone resorption and stimulation of formation by mechanical loading of the modeling rat ulna in vivo. J Bone Miner Res 1995;10:683–9. [PubMed: 7639102]
13. Ikeda T, Kasai M, Suzuki J, Kuroyama H, Seki S, Utsuyama M, Hirokawa K. Multimerization of the receptor activator of nuclear factor-kappaB ligand (RANKL) isoforms and regulation of osteoclastogenesis. J Biol Chem 2003;278:47217–22. [PubMed: 13679377]
14. Ikeda T, Kasai M, Utsuyama M, Hirokawa K. Determination of three isoforms of the receptor activator of nuclear factor-kappaB ligand and their differential expression in bone and thymus. Endocrinology 2001;142:1419–26. [PubMed: 11250921]
15. Inoue D, Reid M, Lum L, Kratzschmar J, Weskamp G, Myung YM, Baron R, Blobel CP. Cloning and initial characterization of mouse meltrin beta and analysis of the expression of four metalloprotease-disintegrins in bone cells. J Biol Chem 1998;273:4180–7. [PubMed: 9461614]
16. Jacobs CR, Yellowley CE, Davis BR, Zhou Z, Cimbala JM, Donahue HJ. Differential effect of steady versus oscillating flow on bone cells. J Biomech 1998;31:969–76. [PubMed: 9880053]
17. Judex S, Zhong N, Squire ME, Ye K, Donahue LR, Hadjiargyrou M, Rubin CT. Mechanical modulation of molecular signals which regulate anabolic and catabolic activity in bone tissue. J Cell Biochem 2005;94:982–94. [PubMed: 15597385]
18. Kamioka H, Honjo T, Takano-Yamamoto T. A three-dimensional distribution of osteocyte processes revealed by the combination of confocal laser scanning microscopy and differential interference contrast microscopy. Bone 2001;28:145–9. [PubMed: 11182371]
19. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 2002;17:210–20. [PubMed: 11811551]
20. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF. Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res 1997;12:2014–23. [PubMed: 9421234]
21. Kim CH, You L, Yellowley CE, Jacobs CR. Oscillatory fluid flow-induced shear stress decreases osteoclastogenesis through RANKL and OPG signaling. Bone 2006;39:1043–7. [PubMed: 16860618]
22. Kinpara K, Mogi M, Kuzushima M, Togari A. Osteoclast differentiation factor in human osteosarcoma cell line. J Immunoassay 2000;21:327–40. [PubMed: 11071251]
23. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH. Sensitivity of osteocytes to biomechanical stress in vitro. Faseb J 1995;9:441–5. [PubMed: 7896017]
24. Knothe Tate, M.; Tami, A.; Nasser, P.; Steck, R.; Schaffler, M. Permeability characteristics of different molecular tracers in loaded and unloaded bone; 47th Annual Meeting of Orthopaedic Research Society; San Francisco, California. 2001; p. 0138
25. Knothe Tate ML, Knothe U, Niederer P. Experimental elucidation of mechanical load-induced fluid flow and its potential role in bone metabolism and functional adaptation. Am J Med Sci 1998;316:189–95. [PubMed: 9749561]
26. Kobayashi Y, Hashimoto F, Miyamoto H, Kanaoka K, Miyazaki-Kawashita Y, Nakashima T, Shibata M, Kobayashi K, Kato Y, Sakai H. Force-induced osteoclast apoptosis in vivo is accompanied by elevation in transforming growth factor beta and osteoprotegerin expression. J Bone Miner Res 2000;15:1924–34. [PubMed: 11028444]
27. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 1999;402:304–9. [PubMed: 10580503]
28. Kusumi A, Sakaki H, Kusumi T, Oda M, Narita K, Nakagawa H, Kubota K, Satoh H, Kimura H. Regulation of synthesis of osteoprotegerin and soluble receptor activator of nuclear factor-kappaB

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ligand in normal human osteoblasts via the p38 mitogen-activated protein kinase pathway by the application of cyclic tensile strain. J Bone Miner Metab 2005;23:373–81. [PubMed: 16133687]

29. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165–76. [PubMed: 9568710]
30. Li YJ, Batra NN, You L, Meier SC, Coe IA, Yellowley CE, Jacobs CR. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. J Orthop Res 2004;22:1283–9. [PubMed: 15475210]
31. Li YJ, Batra NN, You L, Meier SC, Coe IE, Yellowley CE, Jacobs CR. Effects of loading induced oscillatory fluid flow on human marrow stromal cell proliferation and differentiation. J Orthop Res. 2004In Press
32. Liegibel UM, Sommer U, Bundschuh B, Schweizer B, Hilscher U, Lieder A, Nawroth P, Kasperk C. Fluid shear of low magnitude increases growth and expression of TGFbeta1 and adhesion molecules in human bone cells in vitro. Exp Clin Endocrinol Diabetes 2004;112:356–63. [PubMed: 15239020]
33. Lossdorfer S, Schwartz Z, Wang L, Lohmann CH, Turner JD, Wieland M, Cochran DL, Boyan BD. Microrough implant surface topographies increase osteogenesis by reducing osteoclast formation and activity. J Biomed Mater Res A 2004;70:361–9. [PubMed: 15293309]
34. Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, Tempst P, Choi Y, Blobel CP. Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. J Biol Chem 1999;274:13613–8. [PubMed: 10224132]
35. Mizuno A, Kanno T, Hoshi M, Shibata O, Yano K, Fujise N, Kinosaki M, Yamaguchi K, Tsuda E, Murakami A, Yasuda H, Higashio K. Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. J Bone Miner Metab 2002;20:337–44. [PubMed: 12434161]
36. Mueller RJ, Richards RG. Immunohistological identification of receptor activator of NF-kappaB ligand (RANKL) in human, ovine and bovine bone tissues. J Mater Sci Mater Med 2004;15:367–72. [PubMed: 15332601]
37. Owan I, Burr DB, Turner CH, Qiu J, Tu Y, Onyia JE, Duncan RL. Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain. Am J Physiol 1997;273:C810–5. [PubMed: 9316399]
38. Quinn JM, Gillespie MT. Modulation of osteoclast formation. Biochem Biophys Res Commun 2005;328:739–45. [PubMed: 15694408]
39. Rogers A, Eastell R. Circulating osteoprotegerin and receptor activator for nuclear factor kappaB ligand: clinical utility in metabolic bone disease assessment. J Clin Endocrinol Metab 2005;90:6323–31. [PubMed: 16105967]
40. Rubin J, Fan X, Biskobing DM, Taylor WR, Rubin CT. Osteoclastogenesis is repressed by mechanical strain in an in vitro model. J Orthop Res 1999;17:639–45. [PubMed: 10569471]
41. Rubin J, Murphy T, Nanes MS, Fan X. Mechanical strain inhibits expression of osteoclast differentiation factor by murine stromal cells. Am J Physiol Cell Physiol 2000;278:C1126–32. [PubMed: 10837340]
42. Rubin J, Murphy TC, Fan X, Goldschmidt M, Taylor WR. Activation of extracellular signal-regulated kinase is involved in mechanical strain inhibition of RANKL expression in bone stromal cells. J Bone Miner Res 2002;17:1452–60. [PubMed: 12162499]
43. Rubin J, Murphy TC, Zhu L, Roy E, Nanes MS, Fan X. Mechanical strain differentially regulates endothelial nitric-oxide synthase and receptor activator of nuclear kappa B ligand expression via ERK1/2 MAPK. J Biol Chem 2003;278:34018–25. [PubMed: 12824189]
44. Sakai K, Mohtai M, Iwamoto Y. Fluid shear stress increases transforming growth factor beta 1 expression in human osteoblast-like cells: modulation by cation channel blockades. Calcif Tissue Int 1998;63:515–20. [PubMed: 9817947]
45. Saunders MM, Taylor AF, Du C, Zhou Z, Pellegrini VD, Jr. Hj D. Mechanical stimulation effects on functional end effectors in osteoblastic MG-63 cells. J Biomech. 2005

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46. Schett G, Kiechl S, Redlich K, Oberhollenzer F, Weger S, Egger G, Mayr A, Jocher J, Xu Q, Pietschmann P, Teitelbaum S, Smolen J, Willeit J. Soluble RANKL and risk of nontraumatic fracture. Jama 2004;291:1108–13. [PubMed: 14996780]
47. Sipola A, Nelo K, Hautala T, Ilvesaro J, Tuukkanen J. Endostatin inhibits VEGF-A induced osteoclastic bone resorption in vitro. BMC Musculoskelet Disord 2006;7:56. [PubMed: 16839420]
48. Suzuki J, Ikeda T, Kuroyama H, Seki S, Kasai M, Utsuyama M, Tatsumi M, Uematsu H, Hirokawa K. Regulation of osteoclastogenesis by three human RANKL isoforms expressed in NIH3T3 cells. Biochem Biophys Res Commun 2004;314:1021–7. [PubMed: 14751235]
49. Takai, E.; Taboas, J.; Hudson, S.; Tuan, RS. Osteocyte enhancement of angiogenesis in vitro; 53rd Annual Meeting of the Orthopaedic Research Society; San Diego. 2007. p. 0310
50. Tan SD, Kuijpers-Jagtman AM, Semeins CM, Bronckers AL, Maltha JC, Von den Hoff JW, Everts V, Klein-Nulend J. Fluid shear stress inhibits TNFalpha-induced osteocyte apoptosis. J Dent Res 2006;85:905–9. [PubMed: 16998129]
51. Taylor AF, Saunders MM, Shingle DL, Cimbala JM, Zhou Z, Donahue HJ. Mechanically stimulated osteocytes regulate osteoblastic activity via gap junctions. Am J Physiol Cell Physiol 2007;292:C545–52. [PubMed: 16885390]
52. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 2004;15:457–75. [PubMed: 15561602]
53. Trofimov S, Pantsulaia I, Kobyliansky E, Livshits G. Circulating levels of receptor activator of nuclear factor-kappaB ligand/osteoprotegerin/macrophage-colony stimulating factor in a presumably healthy human population. Eur J Endocrinol 2004;150:305–11. [PubMed: 15012615]
54. Wang L, Ciani C, Doty SB, Fritton SP. Delineating bone's interstitial fluid pathway in vivo. Bone 2004;34:499–509. [PubMed: 15003797]
55. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 1998;95:3597–602. [PubMed: 9520411]
56. Yellowley CE, Li Z, Zhou Z, Jacobs CR, Donahue HJ. Functional gap junctions between osteocytic and osteoblastic cells. J Bone Miner Res 2000;15:209–17. [PubMed: 10703922]
57. You J, Yellowley CE, Donahue HJ, Zhang Y, Chen Q, Jacobs CR. Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading-induced oscillatory fluid flow. J Biomech Eng 2000;122:387–93. [PubMed: 11036562]
58. You L, Cowin SC, Schaffler MB, Weinbaum S. A model for strain amplification in the actin cytoskeleton of osteocytes due to fluid drag on pericellular matrix. J Biomech 2001;34:1375–86. [PubMed: 11672712]
59. You LD, Weinbaum S, Cowin SC, Schaffler MB. Ultrastructure of the osteocyte process and its pericellular matrix. Anat Rec 2004;278A:505–13.
60. Zhao S, Zhang YK, Harris S, Ahuja SS, Bonewald LF. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. J Bone Miner Res 2002;17:2068–79. [PubMed: 12412815]
61. Ziegler S, Niessner A, Richter B, Wirth S, Billensteiner E, Woloszczuk W, Slany J, Geyer G. Endurance running acutely raises plasma osteoprotegerin and lowers plasma receptor activator of nuclear factor kappa B ligand. Metabolism 2005;54:935–8. [PubMed: 15988704]

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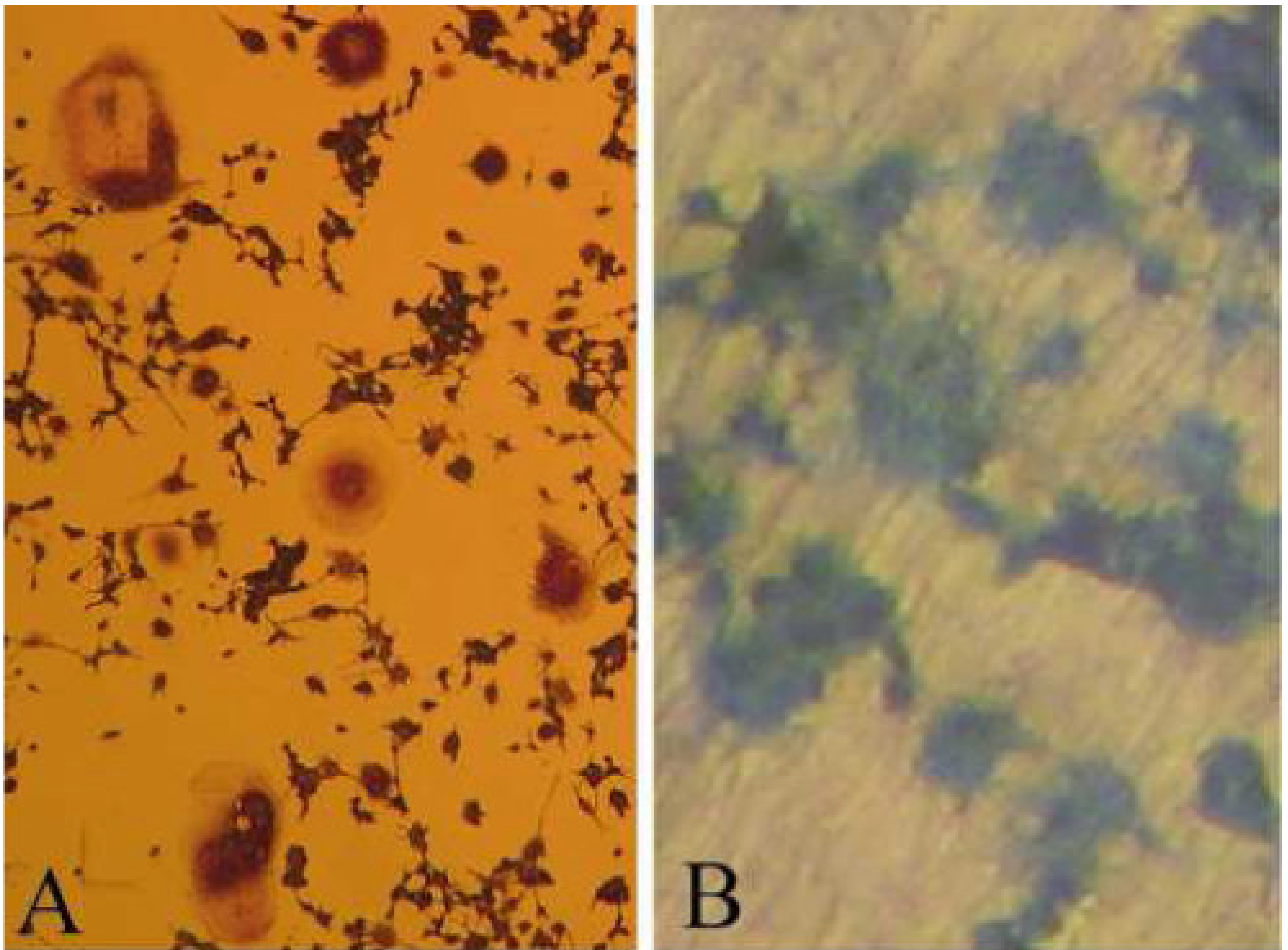

Figure 1.
MLO-Y4 cells induce osteoclast formation and activation when co-cultured with RAW264.7 cells. A) TRAP+ MNCs on 24-well plates at day 9 of co-culturing MLO-Y4 cells and RAW264.7 cells at 500 cells/cm^2 and 2500 cells/cm^2, respectively. B) Pits formed on dentine discs at day 14 after co-culture of MLO-Y4 cells and RAW264.7 cells at the same cell density as described above.

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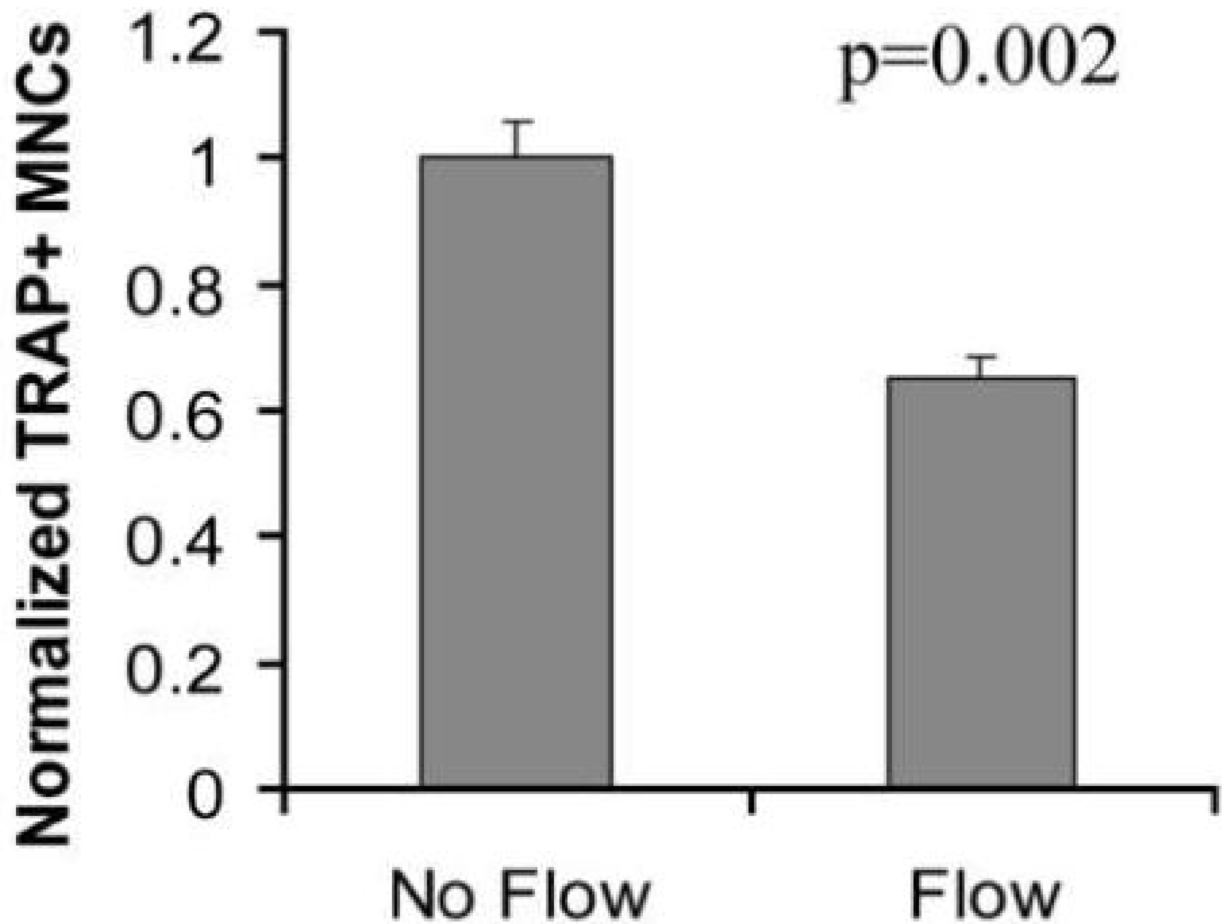

Figure 2.
Osteoclastogenesis in the co-culture of MLO-Y4 and RAW264.7 cells was dramatically inhibited by 38% due to 2 hours of flow exposure of MLO-Y4 cells. Cells were co-cultured for 7 days. TRAP staining was performed at day 9. Osteoclastogenesis were quantified by counting TRAP+ MNCs in 10 randomly chose fields in the center part of slides and values are normalized to controls. The average value corresponding to normalized value 1 is 108 cells. Bars represents means +/− SEM (n = 4 for all groups; p = 0.002).

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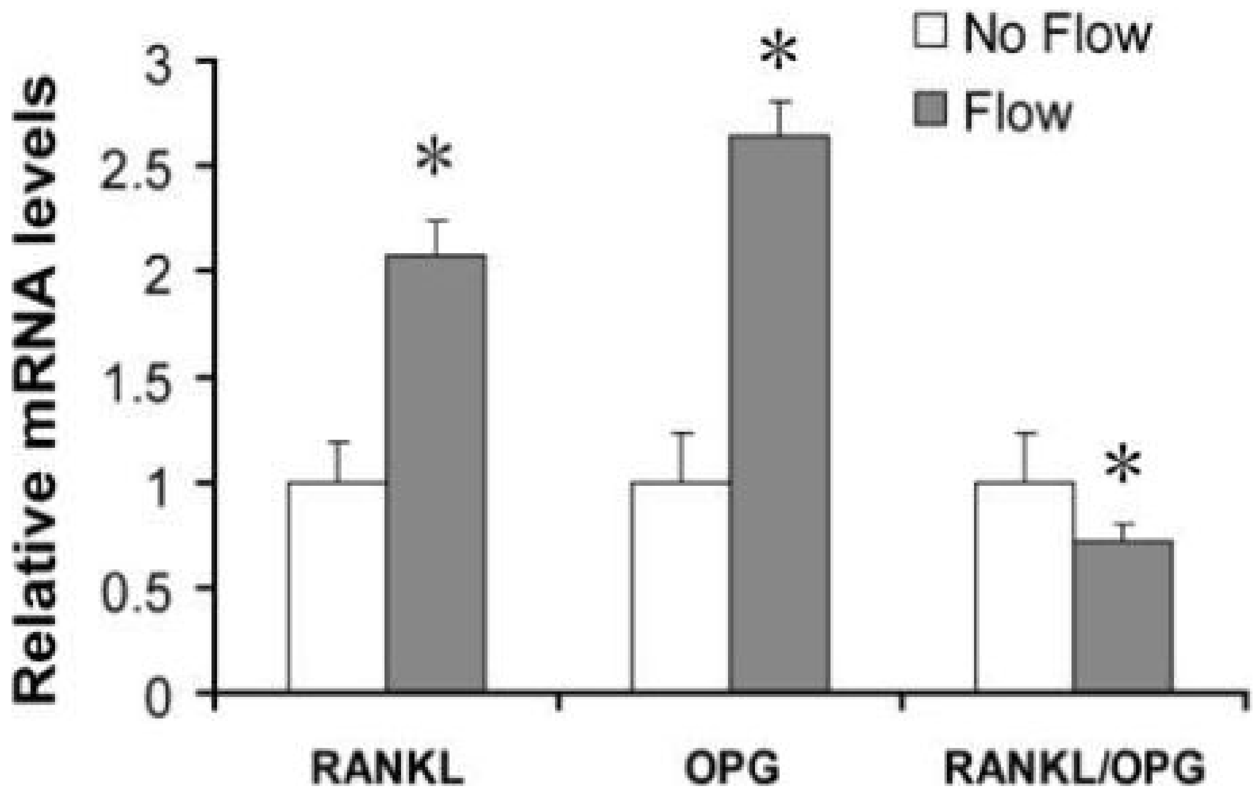

Figure 3.

Cells exposed to 2 hours flow had 2-fold greater RANKL and OPG mRNA levels compared to no flow control group. The ratio of RANKL/OPG was decreased by 29%. Total RNA was isolated immediately following the completion of flow experiment. Bars represents means +/− SEM (n = 4 for all groups; (*) Significant difference between flow group and no flow group, $p < 0.05$).

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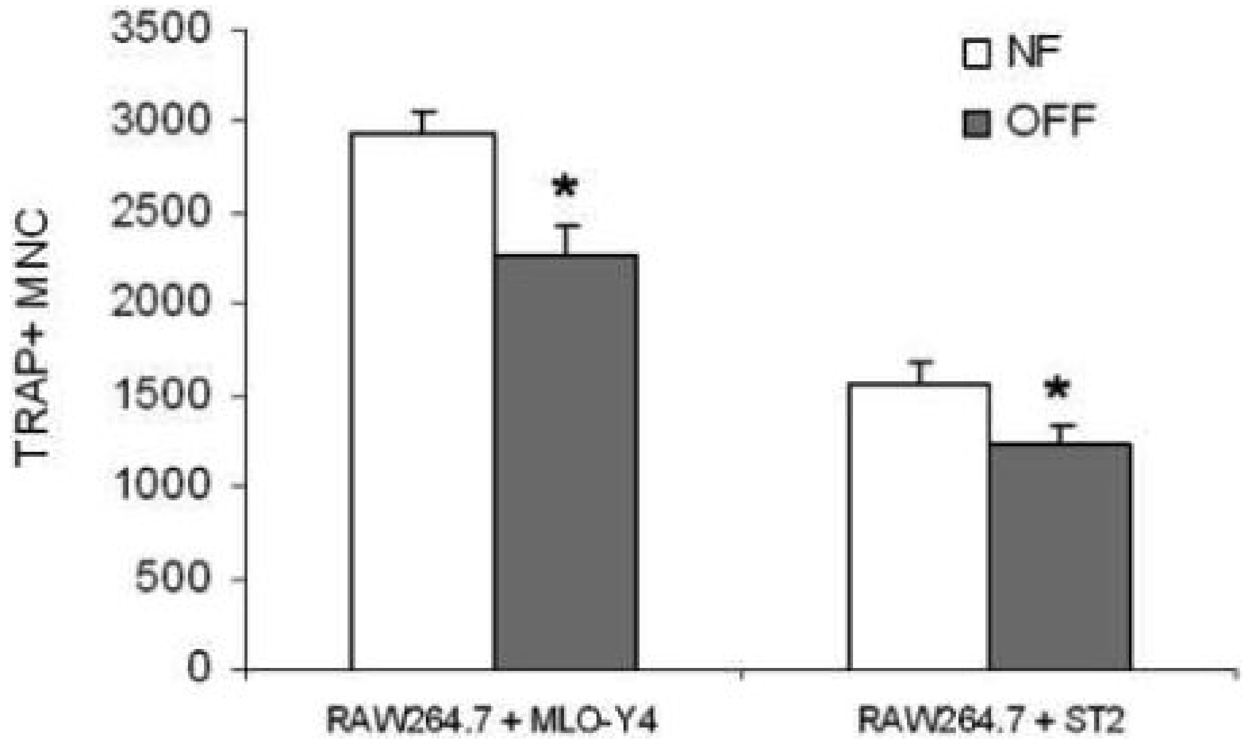

Figure 4.
TRAP+ MNCs formed in two osteoclastogenesis systems: co-culture of MLO-Y4 and RAW264.7 cells, and co-culture of ST2 and RAW264.7 cells. Conditioned medium from MLO-Y4 cells exposed to oscillatory fluid flow or no flow were added to these cultures. Oscillatory fluid flow inhibited osteoclast formation in all of these osteoclastogenesis systems. Bars represents means +/− SEM (n = 4 for all groups; (*) Significant difference between flow group and no flow group, $p < 0.05$).

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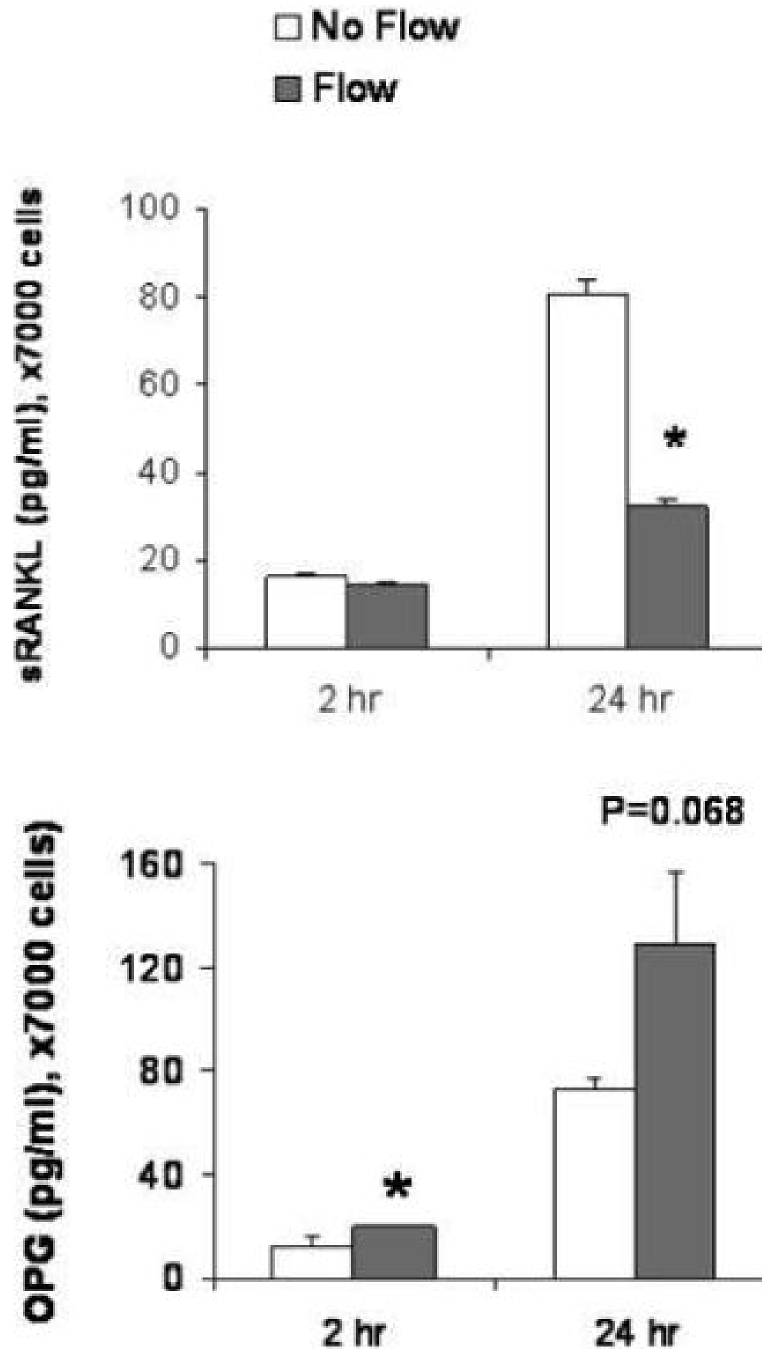

Figure 5.

A. Two hours flow exposure decreased the amount of sRANKL released by MLO-Y4 cells by 10% at 2 hours post flow and 60% at 24 hours post flow. B. MLO-Y4 cells exposed to 2 hours flow released a greater amount of OPG compared with MLO-Y4 cells exposed to no flow at 2 hours post flow (60%) and 24 hours post flow (77%). Bars represents means +/− SEM (n = 4 for all groups; (*) Significant difference between flow group and no flow group, $p < 0.05$)