Oscillatory fluid flow-induced shear stress decreases osteoclastogenesis through RANKL and OPG signaling

Chi Hyun Kim a,b,c,⁎, Lidan You b,c, Clare E. Yellowley d, Christopher R. Jacobs b,c

a Orthopaedic Bioengineering Laboratory, Department of Biomedical Engineering, Yonsei University, Wonju, Kangwon Do, Korea
b Bone and Joint Rehabilitation R&D Center, Department of Veterans Affairs, Palo Alto, CA, USA
c Cell and Molecular Biomechanics Laboratory, Department of Mechanical Engineering, Stanford University, Stanford, CA, USA
d School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology, University of California, Davis, CA, USA

Received 4 October 2005; revised 25 April 2006; accepted 2 May 2006
Available online 24 July 2006

Abstract

Physical activity creates deformation in bone that leads to localized pressure gradients that drive interstitial fluid flow. Due to the cyclic nature of the applied load, this flow is oscillatory by nature. Oscillatory fluid flow (OFF) may lead to positive bone remodeling through effects on both osteoblasts and osteoclasts but its effect on osteoclastogenesis is poorly understood. In this study, the effects of OFF on expression of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG), two important regulators of osteoclast differentiation, were investigated. In addition, its effect on osteoclast formation was quantified. ST-2 murine bone marrow stromal cells were plated on glass slides and cultured with 1,25-dihydroxyvitamin D3 to express RANKL. Cells were exposed to various durations of OFF resulting in a peak shear stress of 1 Pa. Time course and dose–response studies were performed and real-time RT-PCR was used to quantify levels of RANKL, OPG mRNA. ST-2 cells exposed to OFF were also co-cultured with RAW 264.7 monocytes and osteoclast number quantified. Decrease in RANKL/OPG was maximal immediately after end of flow and there existed a significant increase in OPG and decrease in RANKL with increasing load duration of up to 2 h. OFF resulted in a decrease in osteoclast formation by ST-2 cells co-cultured with RAW 264.7 cells compared to co-culture of control (non-loaded) ST-2 cells with RAW 264.7 cells. These results suggest that indeed OFF is a potent regulator of bone remodeling, and that shift towards positive bone remodeling mediated by loading-induced fluid flow may occur via suppression of the formation of osteoclasts.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Oscillatory fluid flow; Mechanotransduction; RANKL; OPG; Osteoclastogenesis

Introduction

Mechanical loading is an important regulator of bone metabolism. There are a number of in vivo models that have been developed to investigate the response of bone to mechanical loading [1–5]. However, in vivo studies present difficulties in determining the cellular signal transduction mechanisms activated by the mechanical input due to the complex cell-level physical signals arising from changes in the pericellular mechanical environment. Thus, many investigators study cellular transduction of physical signals in vitro where controlled physical signals can be applied to bone cells in culture in the absence of other biochemical changes [6–15].

A potent cellular physical signal in the regulation of bone metabolism is loading-induced fluid flow [10–12]. Bone contains a significant fraction of unbound interstitial fluid that surrounds all of the cells found in bone tissue. Motion of this fluid can be driven through externally applied mechanical loading [16], and it has been shown that this fluid flow can be a potent regulator of cell metabolism [10–15]. When bone is functionally loaded, fluid is forced out of regions of high compressive strains and then returns when the load is removed resulting in bone cells being exposed to a dynamic oscillating fluid flow [16]. Theoretical models suggest that information regarding mechanical loading is perceived by bone cells through loading-induced oscillatory fluid flow (OFF) where the flow-induced shear stress magnitude in the lacunar–canalicular
Osteoclasts are the cells responsible for bone resorption. They are formed by fusion of mononuclear progenitors of the monocyte/macrophage lineage. In vitro differentiation of macrophages into osteoclasts requires the presence of two molecules that are expressed by marrow stromal or pre-osteoblastic cells: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) [21,22]. Because pre-osteoblastic cells regulate osteoclastic differentiation, bone resorption and formation are ensured to be tightly coupled. RANKL is a membrane-bound protein that stimulates the osteoclast precursors to commit to the osteoclastic phenotype by binding to its receptor (RANK) on the surface of osteoclast precursors [23]. RANKL is expressed when stimulated by many hormones and cytokines including vitamin D [24–28]. Stromal/osteoblastic cells also express osteoprotegerin (OPG), a decoy receptor that competes with RANK for binding of RANKL [29,30]. OPG binding to RANKL not only blocks osteoclastogenesis but also decreases the survival of pre-existing osteoclasts. The amount of bone resorbed has been reported to be completely dictated by the balance between RANKL and OPG [31,32]. Therefore, it is critical to understand the mechanisms that regulate mechanical signal-induced RANKL and OPG expression.

Although there is a great deal of research currently addressing the mechanism whereby mechanical loading regulates the anabolic behavior of bone cells, very little is known regarding how exercise or physical activity regulates bone resorbing osteoclasts. In this study, we investigated the effects of a physiological cell-level mechanical signal (i.e., dynamic flow-induced shear stress) on the gene expression of RANKL and OPG as well as the resultant induction of osteoclasts.

Materials and methods

RANKL and OPG gene expression study

Cell culture and oscillatory fluid flow

ST-2 (Riken, Japan) murine bone marrow stromal cells were cultured on tissue culture dishes until reaching 80% confluence in alpha-MEM (Invitrogen, Carlsbad, CA) with 10% FBS (HyClone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). They were then placed in a humidified incubator at 37°C and 5% CO₂. Forty-eight hours prior to experimentation, ST-2 cells were subcultured on glass slides (75 × 38 × 1 mm) at a density of approximately 2 × 10⁵ cells/cm² and 10 nM 1α,25-dihydroxyvitamin D₃ (Fluka, Switzerland) added to induce the expression of RANKL. The cultures were continued until the cells have reached approximately 90% confluence.

Forty-eight hours after subculture, the slides with ST-2 cells were placed in custom-built sterile parallel plate flow chambers under sterile conditions. Dynamic oscillatory fluid flow was produced with a Hamilton glass syringe connected in series with rigid walled tubing and a parallel plate flow chamber. The syringe was mounted in and driven by a feedback-controlled linear electromagnetic actuator that can deliver a precise flow rate (EnduraTec, Eden Prairie, MN). The flow rate was monitored using an ultrasonic flow meter (Transonic Systems Inc., Ithaca, NY). The resulting flow profile was a sinusoidal waveform at 1 Hz and a peak shear stress of ±1 Pa [33]. Media for all flow experiments consisted of alpha-MEM with 10% FBS and 1% penicillin/streptomycin. Control cells were also subcultured on glass slides and placed in the parallel plate flow chamber for the same time period as the treated cells but were not exposed to fluid flow. The parallel plate flow chambers were placed in the CO₂ incubator throughout flow exposure to maintain temperature and pH.

Time course study

Oscillatory flow was applied for 1 h and RNA isolated at 4 different time points following cessation of flow (0, 1.5, 24, or 72 h) (n = 4 slides per group). For the 0 h time point group, slides were removed from the parallel plate flow chambers and placed in sterile dishes for immediate RNA isolation. For the 1.5, 24, and 72 h time point groups, slides were removed from the parallel plate flow chambers and placed in sterile dishes. Fresh alpha-MEM with 10% FBS and 1% penicillin/streptomycin was added and cells incubated for another 1.5, 24, or 72 h until RNA isolation.

Dose-response study

Dynamic flow was applied for either 30 min, 1 h, or 2 h (n = 4 slides per group). Immediately after flow, glass slides with cells were removed from the parallel plate flow chambers and placed in sterile dishes for subsequent RNA isolation.

RNA isolation and real-time RT-PCR

Cells were lysed and total RNA isolated using Tri-Reagent (Sigma, St. Louis, MO). The 260/280 absorbance ratio was measured for verification of the purity of RNA. Analysis by quantitative real-time RT-PCR (Perkin Elmer Prism 7900, Applied Biosystems, Foster City, CA) was conducted to determine the relative steady state mRNA levels of RANKL and OPG (Taqman Gene Expression Assays, Applied Biosystems, Foster City, CA). Additionally, rRNA for the housekeeping gene 18S (Taqman Gene Expression Assays, Applied Biosystems, Foster City, CA) was analyzed for each sample. Each RNA sample was analyzed in triplicate.

Osteoclastogenesis study

ST-2 murine bone marrow stromal cells were cultured in tissue culture dishes as above. Forty-eight hours prior to the OFF experiment, ST-2 cells were subcultured on glass slides at a density of 500 cells/cm² and 10 nM 1α,25-dihydroxyvitamin D₃ added for expression of RANKL. Forty-eight hours after subculture, the cells were placed in parallel plate flow chambers and exposed to 2 h of dynamic fluid flow as above (n = 4). The flow rate was selected to yield a peak shear stress of ±1 Pa with a sinusoidal waveform at 1 Hz frequency. Flow media for all OFF experiments consisted of alpha-MEM with 10% FBS and 1% penicillin/streptomycin. Control cells were also subcultured on glass slides (n = 4) and placed in the parallel plate flow chamber for the same amount of time as the treated cells but were not exposed to OFF.

Immediately after flow exposure, the slides were removed from the parallel plate flow chambers and placed in new dishes under sterile conditions. RAW 264.7 monocyte/macrophage cells were added to the slides at a 10:1 ratio.
relative to the ST-2 cells and cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. The ST-2 and RAW 264.7 cells were co-cultured for an additional 4 days and stained for TRAP activity (Sigma, St. Louis, MO). Using a microscope with a 20× objective, TRAP-positive cells with 3 or more nuclei were considered to be osteoclasts and counted by two blinded independent observers.

To observe the resorptive activity of osteoclasts derived from ST-2 and RAW 264.7 co-culture, cells were cultured on 5 mm dentin discs (IDS Ltd.) for 14 days using the conditions described above. At day 15, cells were gently removed from the dentin discs using a toothbrush and the discs were stained with 1% toluidine blue for 2 min, rinsed in distilled water, and air-dried. Resorption pits were visualized by light microscopy.

**Statistics**

RANKL and OPG gene expression levels were normalized against 18S rRNA assayed in the same sample tube. Statistical changes in gene expression and osteoclast number were analyzed using Student’s *t* test with significant difference assumed at *P* < 0.05. Error bars represent standard error.

**Results**

**RANKL and OPG gene expression: time course**

Immediately after exposure to 1 h of flow, the RANKL/OPG ratio was significantly decreased by approximately 60% compared to no flow controls (*P* < 0.05) (Fig. 1). 1.5 h after exposure to 1 h of OFF, the RANKL/OPG ratio was no longer significantly different from no flow controls (*P* > 0.05). With increasing time between cessation of flow and RNA isolation, the difference in RANKL/OPG ratio between OFF and no flow control groups decreased. RANKL/OPG ratio returned to 87% of baseline level after 24 h after 1 h of OFF and 95% of baseline level after 72 h after 1 h of OFF.

**RANKL and OPG gene expression: dose response**

Application of OFF resulted in a dose-dependent increase in OPG mRNA level compared to no flow controls in ST-2 murine stromal cells (Fig. 2). OPG level was significantly increased after 30 min, 1 h, and 2 h of OFF (*P* < 0.05). There was an approximate 5-fold increase in OPG mRNA level after 2 h of OFF compared to no flow controls. OFF also resulted in a dose-dependent decrease in RANKL mRNA level compared to no flow controls (Fig. 2). However, the fold change in RANKL relative to no flow controls was not as dramatic as the fold...
change in OPG level. A significant decrease (approximately 60%) was detected only after 2 h of continuous OFF ($P < 0.05$). Dose-dependent changes in OPG and RANKL mRNA levels resulted in a significant dose-dependent decrease in RANKL/OPG ratio after 30 min, 1 h, and 2 h of OFF compared to no flow controls ($P < 0.05$) of corresponding durations (Fig. 2). The RANKL/OPG ratio being expressed by ST-2 cells was drastically decreased to 10% of control after 2 h of OFF.

### Osteoclastogenesis

Co-culture of ST-2 murine stromal cells and RAW 264.7 monocyte/macrophage cells resulted in formation of TRAP-positive multinucleated cells. Furthermore, these cells were functional in that they produced resorption pits when cultured on dentin slices as representative of osteoclasts (Fig. 3a). However, co-culture of RAW cells with ST-2 cells that were exposed to 2 h of OFF resulted in significantly fewer osteoclasts (54% decrease, $P < 0.05$) compared to co-culture of RAW cells with ST-2 cells that were not exposed to flow (Fig. 3b).

### Discussion

In this study, we investigated the anti-resorptive effects of mechanical stimulation using an in vitro model. Specifically, oscillatory fluid flow (OFF), a cell-level physical signal that occurs with cyclic tissue loading, was applied while assaying changes in the expression of RANKL and OPG mRNA, two important regulators in osteoclast formation, in murine stromal cells. The resulting change in RANKL/OPG ratio in the stromal cells was then directly related to a change in osteoclast differentiation by monocyte/macrophage cells in co-culture.

Application of loading-induced oscillatory fluid flow for only 1 h significantly decreased the RANKL/OPG mRNA ratio in stromal cells compared to control groups that were not exposed to flow. This decrease in RANKL/OPG ratio after mechanical stimulation is consistent with previous studies that utilized a substrate deformation device to deliver a direct mechanical strain to primary murine stromal cells [9,34]. It is interesting to note that in contrast with substrate strain that only affected RANKL mRNA, oscillatory fluid flow induced a decrease in RANKL as well as an increase OPG mRNA. Also, substrate strain requires between 6 h and 3 days of continuous cyclic stimulation to have an effect where we observed a response within 30 min to 2 h of stimulation (i.e., 1800–7200 loading cycles). Furthermore, a physiological level of OFF-induced shear stress (compared to a supraphysiological level of mechanical strain) was able to induce a similar decrease in RANKL [19,20]. These findings suggest that OFF-induced shear stress may be a more potent stimulator of RANKL and OPG in bone cells compared to substrate deformation.

The drop in RANKL/OPG ratio immediately after 1 h of OFF was lost with time after cessation of flow and recovered to 87% of baseline level after 24 h and 95% after 72 h. This suggests that the effect of mechanical loading on RANKL/OPG signaling in bone occurs immediately and the effect is transient. A longer loading duration resulted in a greater drop in RANKL/OPG ratio, suggesting that the effects of mechanical loading on anti-resorptive events are dose-dependent and may be cumulative. However, the RANKL/OPG ratio was diminished to 10% of baseline level after a dynamic loading duration of only 2 h. Interestingly, the decrease in RANKL/OPG ratio at each loading duration was due to both a decrease in RANKL mRNA level and an increase in OPG mRNA level. This combined effect acted synergistically to significantly decrease the RANKL/OPG ratio by approximately 60% compared to no flow controls after only 30 min of flow. Rubin and colleagues have shown that osteoclast formation results in a drop in RANKL/OPG ratio due to a significant change in RANKL expression but not OPG [34]. Our results indicate that OPG mRNA is upregulated by 50% after 30 min of OFF and by 5-fold after 2 h of OFF. Although both loading approaches result in similar outcomes in that they both decrease RANKL/OPG ratios, these results support the idea that bone cells may respond differently to different mechanical patterns and flow-induced shear stress may regulate RANKL and OPG expression via a different intracellular mechanotransduction pathway than substrate deformation.

It has been shown that a change in RANKL/OPG ratio has a direct impact in osteoclast formation in vitro [32]. We were able to confirm that the change in RANKL/OPG we observed in response to flow exposure directly affects osteoclast formation. Consistent with substrate deformation studies that report a 50% decrease in osteoclastogenesis after 3 days of mechanical stimulation [34], when stromal cells exposed to 2 h of OFF were co-cultured with RAW 264.7 cells for 4 days, there was a 54% decrease in the number of osteoclasts compared to RAW cells co-cultured with stromal cells not exposed to OFF. In our dose–response study, stromal cells exposed to OFF for 2 h demonstrated a significant increase in OPG expression and a significant decrease in RANKL expression compared to control cells. Because RANKL expression on stromal/osteoblastic cells is necessary for osteoclastic differentiation of pre-osteoclastic cells, the downregulation of RANKL appears to have played a major role in decreasing the number of osteoclasts in the co-culture system. In addition, the formation of osteoclasts is further reduced due to the significant upregulation of the decoy receptor, OPG, which blocks the differentiation of pre-osteoclastic cells into osteoclasts by inhibiting the binding of RANK to RANKL.

In this study, the effects of OFF on RANKL and OPG were quantified at the mRNA level. We believe that changes at the mRNA level will directly result in changes at the protein level similar to the study by Fan et al. who also utilized ST-2 stromal cells [35]. Nonetheless, changes at the protein level need to be quantified in order to be conclusive.

In summary, dynamic fluid flow that occurs with loading has been demonstrated to be a potent regulator of osteoclast formation and this cell-level physical stimulus inhibits osteoclastogenesis through an upregulation of OPG and a downregulation of RANKL. Numerous studies have established the anabolic effect of mechanical stimulation and its important role in osteoblastic bone formation. However, results presented here suggest that mechanical loading can also prevent negative bone remodeling via suppression of bone resorbing osteoclasts and that loading-induced fluid flow may be a major contributor of this suppression.
Acknowledgments

This study was supported by NASA Grant NAG2-1601 (CRJ), NIH AR45989 (CRJ), and Yonsei University Research Fund of 2005 (CHK). We gratefully acknowledge Ms. Christina Lee for her help with culture of the RAW 264.7 cells, Dr. Janet Rubin and Dr. Xian Fan for their expertise in vitamin D-induced RANKL expression, and Dr. Simon Fox for his help with TRAP staining.

References

[31] Hofbauer LC, Gori F, Rigsby BL, Lacey DL, Dunstan CR, Spelsberg TC, et al. Stimulation of osteoprotegerin ligand and inhibition of osteo-
protegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteo-