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# Mechanical stimulation effects on functional end effectors in osteoblastic MG-63 cells

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**Abstract**

Receptor activator of Nf-κB ligand (RANKL) and osteoprotegerin (OPG) have been implicated in bone metabolism. Specifically, the balance of these factors in conjunction with receptor activator of Nf-κB (RANK) is believed to be key in determining the rate of osteoclastogenesis and the net outcome of bone formation/resorption. While it is well accepted that mechanical loading in vivo affects bone formation/resorption and that alterations in the responsiveness of bone cells to mechanical loading have been implicated in metabolic bone diseases, the effect of in vitro mechanical loading on osteoblastic production of OPG and RANKL has not been extensively studied. Thus, in the current study, we developed an in vitro model to load human osteoblasts and studied levels of OPG, RANKL, $PGE_2$ and macrophage colony stimulating factor (M-CSF). We hypothesized that stimulating osteoblastic cells would increase the release of soluble OPG relative to RANKL favoring a bone-forming (and resorption-inhibiting) event. To accomplish this, we developed a small-scale loading machine that imparts via bending, well-defined substrate deformation to bone cells cultured on artificial substrates. Following 2 h of loading and a 1 h incubation period, media was collected and levels of soluble OPG, RANKL, $PGE_2$ and M-CSF were quantified using ELISA and western blotting. We found that mechanical loading significantly increased soluble OPG levels relative to RANKL at this 3 h time point. Levels of soluble and cellular RANKL detected were not significantly affected by mechanical stimulation. The relative shift in abundance of OPG over RANKL associated with applied mechanical stimulation suggests the soluble OPG:RANKL ratio may be important in load-induced coupling mechanisms of bone cells.


*Keywords:* Mechanotransduction; Substrate deformation; Osteoprotegerin; RANK ligand; Bone

## 1. Introduction

Osteoclasts and osteoblasts are uniquely linked. Research suggests that osteoclasts rely on both juxtacrine and soluble factors from osteoblasts for formation, function and apoptosis. For instance, models examining osteoclastogenesis have concluded that osteoclastogenesis is possible only in the presence of cells of the stromal and osteoblast lineages (Suda et al., 1996, 1999). More recently it has been determined that these cell types express receptor activator of Nf-κB ligand (RANKL) (also known as OPGL, ODF and TRANCE) which, in the presence of macrophage colony stimulating factor (M-CSF) (also known as CSF-1) is both necessary and sufficient to induce osteoclastogenesis in a mouse model (Shalhoub et al., 1999). While the M-CSF is required to commit the cells to the osteoclast lineage, RANKL, expressed by mature osteoblasts binds to the preosteoclast via receptor activator of Nf-κB (RANK) generating a functional osteoclast. Osteoprotegerin (OPG) is a soluble decoy receptor produced by osteoblast/stromal cells that inhibits osteoclastogenesis (Shalhoub et al.,

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1999) by binding directly to RANKL and blocking the ligand from RANK. These three molecules RANK, RANKL and OPG are believed to be important end effectors that have an outcome on bone formation and resorption (Aubin and Bonnelye, 2000; Hofbauer, 1999).

For bone to adapt, bone cells must be sensitive to loading effects. Thus, load induced from the global environment reaches the cellular level in the form of a biophysical signal, which affects the cells to bring about a response (mechanotransduction). Mechanotransduction studies have found that bone cells can respond to a variety of endogenously occurring signals including stretch, chemotransport, electrical effects and fluid flow. With respect to the latter, it has been hypothesized that bone cells embedded in and around mineralized matrix are connected via fluid-filled canalicular networks. Fluid flow systems have been characterized and we, and others, have employed them to expose cells (Hung et al., 1995, 1996; Jacobs et al., 1998; Saunders et al., 2001, 2003; You et al., 2000, 2001) to physiologic levels of fluid flow (0–30 dyne/cm$^2$).

While fluid flow models predict and mimic the movement of fluid through canalicular networks to be oscillatory under dynamic conditions, cells on endosteal and periosteal bone surfaces, such as bone lining cells or surface-residing osteoblasts, are also likely to be subjected to substrate deformation as the bone bends upon loading. In this model, effects of fluid movement would be secondary to the loading induced by the bone (substrate) deformation and the stimulation of these surface-residing cells may be involved in the orchestration/activation of the surface bone formation/resorption events (Everts et al., 2002). Thus, the development of mechanotransduction models that examine the role of surface-residing osteoblastic cells and substrate deformation are needed to help elucidate the mechanisms involved in bone formation/resorption and the role mechanical loading plays in this process. This has application to surface bone turnover events encountered in implant design, bone/implant interfacing and implant-induced osteolysis. Therefore, in the current study we developed a loading machine to subject human osteoblastic cells to mechanical stimulation via substrate deformation. We utilized MG-63 osteoblastic cells as a model for surface-residing osteoblasts. We measured functional (protein) levels of OPG, RANKL, $PGE_2$ and M-CSF. OPG, RANKL, $PGE_2$ and M-CSF were quantified in their soluble forms with ELISA, while RANKL was additionally quantified in its total and membrane-bound forms with western blotting techniques. We hypothesized that OPG and RANKL may be important regulators of load-induced bone formation/resorption by coupling osteoblastic and osteoclastic mechanisms. Moreover, because it is well known that bone can adaptively respond to very low cycle numbers and frequencies of mechanical stimulation (Rubin and Lanyon, 1984; Mosely et al., 1997), we wanted to study early responses to stimulation, i.e., stimulation-induced secretory changes.

## 2. Methods

The purpose of the current study was three-fold. First, to simulate the environment experienced by surface-residing osteoblasts we developed a small-scale loading machine and environmental chamber to impart reproducible bending to osteoblastic cells seeded on deformable substrates. Second, we quantified functional activity of OPG, RANKL and $PGE_2$ as a result of this physiologic substrate deformation. Third, we correlated the differential activity of OPG and RANKL with M-CSF. $PGE_2$ was selected because it is a known intermediate responder in bone remodeling and stimulation in osteoblastic cells (Imamura et al., 1990; Jee et al., 1985). M-CSF is produced by stromal cells and osteoblasts and along with RANKL is a prerequisite for the differentiation of osteoclast progenitors to mature bone resorbing osteoclasts. M-CSF is known to be involved in tissue remodeling and has been suggested to function independently of macrophages involved with inflammation (Cecchini et al., 1994; Stanley, 1994). Correlating RANKL and OPG with M-CSF activity should permit us to assess if the cellular response is mediated by the loading regime or an inflammatory response.

### 2.1. Design, fabrication and characterization of small-scale loading system

The loading machine developed is shown (Fig. 1). Three-point bending fixtures were machined from Delrin®. The lower contacts are at a fixed distance of 50.8 mm and are recessed 12.7 mm into a reservoir. This enabled the cells to be completely submersed in medium during testing and the reservoir was placed in a sterile, Lexan® environmental chamber to control for physiologic conditions (Fig. 1). Chamber heat is provided by eight miniature proportionally controlled heaters (Hytek). Two pins of the heater connect to a 3 A (13.8 VDC) power supply and two pins connect to a temperature set resistor. Studies were conducted to verify that the microheaters in combination (all with 1.1 kΩ resistors) reached the required internal chamber temperature of 37 °C. Each heater with a 1.1 kΩ resistor maintained a maximum surface temperature of 57.5 °C at 5 min (Fig. 2a). After that, no fluctuation in temperature was recorded with experiments conducted in ambient air. Furthermore, the eight resistors (2 per chamber side) enabled the internal environment to reach 36.3 °C after 1 h of heating (measured at a depth consistent with the