OSTEOCYTES SUBJECTED TO PULSATING FLUID FLOW REGULATE THE PROLIFERATION AND DIFFERENTIATION OF OSTEOBLASTS

** INTRODUCTION:** Bone is a highly mechanosensitive tissue, able to adapt functionally to variety of complex mechanical stimuli. One type of mechanical stimulus, pulsating fluid flow (PFF), mimics the magnitude and duration of *in vivo* loading of osteocytes [1, 2]. The lacunocanalicular system of bone consists of osteocytes residing in lacunae and sending off processes through canaliculi, thus enabling osteocyte contact. Special pericellular fluid bathes the osteocyte, so a mechanical stimulus is transmitted, through direct contact and the ensuing flow of pericellular fluid, to the mechanosensitive osteocytes. While osteocytes are thought to be the most mechanically sensitive bone cell population [1], osteoblasts and periosteal fibroblasts are two other bone cells that are thought to be not as mechanically sensitive [2] but may play important roles in bone mechanoregulation. Periosteal fibroblasts contain osteoblastic precursor cells that are hypothesized to be even less mechanically sensitive than osteoblasts.

Because cells communicate through gap junctions [3], it is unclear whether important soluble factors are released by osteocytes in response to PFF that in turn influence the proliferation and/or differentiation of neighboring cells. Previously we have demonstrated that osteocytes subjected to PFF produce nitric oxide (NO), a mediator of bone formation *in vivo* [4]. In this study we tested the hypothesis that three different bone cell populations—osteocytes, osteoblasts, and periosteal fibroblasts—subjected to PFF would produce conditioned medium (CM) containing soluble factors that modulate the proliferation and/or differentiation of cultured osteoblasts and periosteal fibroblasts, with osteocyte PFF CM producing the strongest effect.

**METHODS:** *Cell isolation and culture:* Osteocytes (OCYs), osteoblasts (OBs), and periosteal fibroblasts (PFs) from 18d fetal chinchilla calvaria were isolated immunomagnetically utilizing a monoclonal antibody Mab OB7.3 [5]. Mechanical stimulation: OCYs, OBs, and PFs were cultured on slides (4.0x10^5 cells) in the presence and absence of L-NAME, a nitric oxide synthase inhibitor. Concurrently, OBs and PFs were cultured in 96-well plates (1.0x10^5 cells/well) and 24-well plates (3.0x10^5 cells/well). After 24h, slides were refreshed with medium and subjected to 1h of PFF or static control. PFF was generated using a parallel-plate flow chamber system (0.7 Pa fluid shear, 5 Hz) [2]. CM was collected after 1h and was used for cell proliferation and alkaline phosphatase (ALP) activity assays. *Cell proliferation:* CM was plated in the 96-well plates (100 μl/well in triplicate). After 0, 1, 2, and 5 days, cell proliferation was quantified with a fluorescent DNA assay. *ALP activity:* CM was plated in the 24-well plates at 100%, 50%, and 10% (500 μl/well). After 48h, ALP was quantified with a p-nitrophenyl phosphate colorimetric assay, and protein content was measured. *Statistics:* One-way ANOVA with Tukey-Kramer post test and two-tailed t-tests were used with p<0.05 considered statistically significant.

**RESULTS:** Osteocyte PFF CM significantly inhibited the proliferation of cultured osteoblasts (Fig. 1A). Inhibition was statistically significant at each time point, i.e. days 1, 2, and 5. L-NAME treatment decreased the inhibitory effects of OCY PFF CM on osteoblast proliferation (Fig. 1B). Inhibition by OCY + L-NAME PFF CM compared to OCY + L-NAME CO CM only became statistically significant after 5 days. OCY PFF CM produced significantly stronger inhibition of osteoblast proliferation than PFF CM from osteoblasts or periosteal fibroblasts. OCY + L-NAME PFF CM produced a magnitude of inhibition similar to OB PFF CM and PF PFF CM. By day 2, OCY PFF CM inhibited OB proliferation 3.7-fold, whereas all other types of CM—including L-NAME-treated OCY PFF CM—produced no higher than 2-fold inhibition. By day 5, OCY PFF CM produced 17.8-fold inhibition, whereas all other CM types only produced 4-fold inhibition. OCY PFF CM also significantly inhibited PF proliferation. OCY + L-NAME PFF CM did not significantly inhibit PF proliferation at any time point.

Osteoblasts cultured for 48h in the presence of 100% OCY PFF CM experienced a significant upregulation in ALP activity (Fig. 2A). 50% OCY PFF CM did not produce a significant effect on ALP. One-way ANOVA produced p=0.0007. L-NAME eliminated this ALP stimulation (Fig. 2B). 100% OCY + L-NAME PFF CM produced an increase in ALP, and 50% OCY + L-NAME PFF CM produced a slight increase in ALP, however neither increase was statistically significant.

**DISCUSSION:** In this study we examined the effects of PFF on three different bone cell populations. We found that osteocyte PFF CM significantly inhibited osteoblast and periosteal fibroblast proliferation. Osteoblast and periosteal fibroblast PFF CM slightly inhibited osteoblast proliferation, but these types of CM produced no significant effect on periosteal fibroblast proliferation. Treatment of osteocytes, osteoblasts, and periosteal fibroblasts with the NO synthase inhibitor L-NAME decreased the inhibitory effects of PFF CM. This suggests that a change in the release of NO is partially responsible for the inhibitory effect of PFF on osteoblast and periosteal fibroblast proliferation. We also found that osteocyte PFF CM stimulated the differentiation of osteoblasts as measured by an increase in ALP activity, and treatment of osteocytes with L-NAME decreased the stimulatory effects of osteocyte PFF CM on osteoblast differentiation. We conclude that osteocytes subjected to PFF inhibit the proliferation but stimulate the differentiation of osteoblasts *in vitro.* Moreover, NO may play an important role in this effect, as preventing NO release increases osteoblast proliferation and decreases differentiation. Our results provide further evidence that the osteocyte is more sensitive to PFF than either the osteoblast or the periosteal fibroblast. This work represents the first time that the effects of conditioned medium from primary osteocytes, as opposed to osteocyte-like cell lines, have been examined. Taken together, the results of the present study provide valuable insight into the local environmental changes that occur in the lacunocanalicular network during bone loading.


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