ELSEVIER

Journal of Orthopaedic Research 22 (2004) 1283–1289

Journal of Orthopaedic Research

www.elsevier.com/locate/orthres

Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation

Ying Jun Li [a,b,*], Nikhil N. Batra [a,b], Lidan You [a,b], Stephen C. Meier [a,b], Ian A. Coe [a,b], Clare E. Yellowley [c], Christopher R. Jacobs [a,b]

[a] *Palo Alto Veterans Administration Medical Center, Palo Alto, CA 94304, USA*
[b] *Department of Mechanical Engineering, Stanford University, Stanford, CA 94305, USA*
[c] *Department of Veterinary Medicine, University of California Davis, Davis, CA 95616, USA*

Abstract

Mechanical loading is an important regulator of bone formation and bone loss. Decreased osteoblast number and function are important cellular mechanisms by which mechanical disuse leads to decreased bone formation. Decreased osteoblast number may be a result of decreased osteoprogenitor proliferation, differentiation, or both. However, the effects of cellular level physical signals on osteoprogenitors are not well understood. In this study, we examined the effects of loading induced oscillatory fluid flow (OFF), a potent regulator of osteoblastic cell function, on marrow stromal cells (MSCs). MSCs subjected to OFF exhibited increased intracellular Ca^{2+} mobilization. In addition, MSCs exhibited increased proliferation and increased mRNA levels for osteopontin and osteocalcin genes. Collagen I and core binding factor 1 mRNA levels did not change. MSCs subjected to OFF also exhibited decreased alkaline phosphatase activity. These results suggest that MSCs are mechanosensitive and that Ca^{2+} may play a role in the signaling pathway.

Keywords: Mechanical loading; Stromal cells; Osteoprogenitors; Osteoblasts; Differentiation; Calcium imaging

Introduction

Mechanical loading is an important regulator of bone metabolism. Skeletal unloading results in increased bone resorption [23], decreased bone mineral density [23], decreased bone formation [4,42], decreased bone growth and strength [39], and reduced mineralization [4]. Changes in osteoblast number and function are important cellular mechanisms by which mechanical disuse leads to decreased bone formation. Mechanical unloading has been associated with decreased osteoblast number [2,4,42], reduced osteoblast size [42], and decreased mineral apposition rate [4]. Two potential sources have been identified for new bone forming osteoblasts: bone lining cells [3,38] and osteoprogenitors found in the bone marrow stroma [2,19,24,37,46]. However, experimental evidence suggests that bone lining cells have lost the ability to proliferate [3,38]; hence, under long-term steady state conditions, osteoblast numbers are regulated by the rate of proliferation and differentiation of osteoprogenitors. Therefore, altering osteoprogenitor proliferation, differentiation, or both may be important mechanisms by which mechanical signals regulate bone formation.

Currently, little is understood about the mechanical regulation of osteoprogenitors at the cellular and molecular levels. We hypothesized that oscillatory fluid flow (OFF) is an important regulator of osteoprogenitors. Mechanical loading induces fluid flow in bone [25,41], and this flow is a potent regulator of osteoblastic and osteocytic cells [6,16,21,33,44]. We found the effects of OFF on osteoblastic (MC3T3-E1, hFOB) and osteocytic (MLO-Y4) cells to include intracellular Ca^{2+} mobilization [16], prostaglandin E_2 (PGE_2) release [33], increased osteopontin (OP) gene expression [44], increased mitogen-activated protein (MAP) kinase activity [44], and inhibition of NF-κB DNA binding activities [21]. While the flow regime experienced by the osteoprogenitors in the bone marrow is not known quantitatively, any flow resulting from physical loading will be

* Corresponding author. Address: C/o Christopher Jacobs, Department of Mechanical Engineering, Stanford University, BME 233 Durand, Mail Code 4038, Stanford, CA 94305, USA. Tel.: +1-650-736-0802; fax: +1-650-725-1587.
E-mail address: yingli@rrdmail.stanford.edu (Y.J. Li).

0736-0266/$ - see front matter © 2004 Orthopaedic Research Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.orthres.2004.04.002

oscillatory in nature. In addition, osteoprogenitors may be regulated by loading induced OFF once they are recruited to sites of bone formation.

We chose marrow stromal cells (MSCs) based on the substantial evidence that they contain osteoprogenitors [11,18,30,32]. Friedenstein first reported that a small number of adherent cells from bone marrow were able to differentiate into bone and cartilage-like colonies [11]. Through selective culturing techniques that isolate progenitor cells from other marrow cells, MSCs can now be enriched considerably from bone marrow aspirates [18,30]. Previously MSCs were isolated from donor rats, loaded into transplantation vehicles, and placed subcutaneously in recipient rats. Subsequently, the donor cells in the transplantation vehicles formed mineralized bone [22]. In addition, when transplanted into the marrow cavity of the recipient, donor MSCs migrated throughout the body and became long-term precursors for bone tissue [29].

The purpose of this study was to examine the effects of OFF on MSCs in terms of early signal transduction and downstream changes. The experiments consisted of quantifying intracellular Ca^{2+} mobilization, cell proliferation, gene expression, and alkaline phosphatase (ALP) activity. Ca^{2+} is an important signaling molecule that has an important role in the bone adaptation to mechanical loading [16,44]. In osteoblastic cells, You et al. [44] demonstrated that OFF regulated OP gene expression through intracellular Ca^{2+} release via inositol 1,4,5-trisphophate (IP_3) sensitive channels. Additionally, we quantified the proliferation rate of MSCs exposed to OFF because MSC proliferation rate directly affects the number of osteoprogenitors available for recruitment into osteoblasts. Furthermore, MSC differentiation was studied by considering gene expression and ALP activity. The mRNA levels of four osteoblast marker genes were examined: core binding factor 1 (Cbfa1), osteopontin (OP), osteocalcin (OSTC), and type I collagen (Col I). Cbfa1 is a necessary transcriptional activator of osteoblast differentiation and is localized to the skeletal system [43]. OP is a mineral-binding, non-collagenous matrix protein and is thought to promote osteoblast attachment and migration [10,46]. OSTC is a non-collagenous protein found exclusively in the bone extracellular matrix [7] and is thought to be upregulated at the onset of mineralization [26]. Col I is the most abundant extracellular protein in bone and is expressed during all stages of osteoblast development [10,31]. Finally, ALP activity is commonly found to precede bone matrix mineralization and is used to assess osteoblast activity [5,18,19,24,26,28,30,37]. ALP activity is also a marker of the various stages of osteoblast differentiation, including the differentiation of MSCs into osteoblast-like cells [18,30]. The demonstration that a cellular-level physical signal has the potential to regulate osteoprogenitor proliferation and differentiation is a novel and significant finding. Our strategy of examining both early signaling and late downstream changes is suggestive of a causal link; however, demonstrating a specific biochemical mechanism will require extensive follow-on studies of cellular signaling that are beyond the scope of the current investigation.

Materials and methods

Experimental design

The effects of OFF on MSCs were studied using Ca^{2+} mobilization, cell proliferation, gene expression, and ALP activity. The Ca^{2+} experiment was used to determine if OFF could produce an intracellular $[Ca^{2+}]$ increase, as had been observed in bone cells. The effect of OFF on MSC proliferation was studied because MSC proliferation directly affects the number of available osteoprogenitors. The mRNA levels of Cbfa1, OP, OSTC, and Col I were utilized as markers of altered gene expression reflective of osteogenic differentiation. In addition, ALP activity was assayed to provide information on potential osteogenic changes occurring at the protein level. ALP activity is a late marker of osteogenic differentiation, and MSCs exhibit significant levels of ALP activity only in the presence of media that supports mineralization [18,30]. Therefore, MSCs were cultured in osteogenic media, according to protocols described in literature [18].

Materials

Reagents were purchased from the following companies: *BioWhittaker* (Walkersville, MD)—cryopreserved human mesenchymal stem cells (MSCs), Dulbecco's Modified Eagle Media (DMEM), fetal bovine serum (FBS), L-glutamine. *Gibco/Life Technologies* (Rockville, MD)—α-MEM, penicillin/streptomycin, trypsin/EDTA, phosphate buffered saline (PBS). *Sigma* (St. Louis, MO)—dexamethasone, β-glycerophosphate, poly-l-lysine, alkaline phosphatase kit 104. *Qiagen* (Valencia, CA)—RNeasy Mini Kit. *Wako Chemicals* (Richmond, VA)—ascorbic acid-2 phosphate. *Molecular Probes* (Eugene, OR)—Fura-2 AM, Fura-2 AM calibration kit, Absolute-S SBIP Cell Proliferation Assay Kit (A-23150). *Pierce* (Rockford, IL)—BCA Protein Assay Kit.

Cell culture

MSCs were purchased from a commercial source (BioWhittaker), where MSCs were obtained from healthy human volunteers by bone marrow aspiration followed by density gradient centrifugation and selective culturing techniques [18,30]. These cells include mesenchymal progenitors and are able to differentiate into osteoblastic cells [30]. At our lab, cells were thawed and cultured at standard conditions of 37 °C, 5% CO_2, and 100% humidity. Complete growth media consisted of DMEM, 10% FBS, 2% L-Glutamine, and 1% penicillin/streptomycin. Fresh complete growth media was exchanged every 3–4 days, and cells were subcultured every 7 days using 0.05% trypsin—0.53 mM EDTA. Cells were between passage numbers 2–8. For the calcium imaging experiments, cells were cultured on UV transparent quartz slides for 3 days and subjected to OFF. For the 5-bromo-2′-deoxyuridine (BrdU) incorporation, gene expression, and ALP activity experiments, all cells were cultured in complete growth media on 0.01% poly-l-lysine coated glass slides for 3–5 days, then one group was subjected to OFF while the other group received no flow. Osteogenic media consisted of complete growth media plus osteogenic supplements: 5 nM dexamethasone, 0.025 mM ascorbic acid-2 phosphate, and 5 mM β-glycerophosphate.

Oscillatory fluid flow (OFF)

Cells were subjected to OFF in a parallel plate flow chamber [16]. The flow regimes were selected based on previous studies of OFF in osteoblastic cells [6,16,21,33,44]. Briefly, cells were cultured on slides and placed against a polycarbonate manifold. The flow channel

geometry was $38\times10\times0.28$ mm for the calcium imaging experiments and $60\times24\times0.28$ mm for all other experiments. Since the Reynolds number for the flow rate used was below 50 and well within the laminar flow regime, the shear stress on the cells could be calculated using Poiseuille's equation. The chamber was sealed, and fluid flow was delivered to the inlet by gastight Hamilton syringes mounted between electromechanical actuators (ElectroForce Actuator, EnduraTec, Minnetonka, MN). The actuators were controlled for maximum displacement, waveform, and frequency using Wintest software (EnduraTec). Flow parameters were verified using a high-frequency ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY).

Calcium imaging

Intracellular Ca^{2+} levels were quantified using a ratiometric imaging technique with the Ca^{2+} sensitive dye Fura-2 (Fura-2). Fura-2 exhibits a shift in absorption when illuminated with UV light such that its emission intensity increases with Ca^{2+} concentration when excited at 340 nm and decreases with Ca^{2+} concentration when excited at 380 nm. The ratio of the emission intensities F_{340}/F_{380} is a quantitative measurement of intracellular calcium $[Ca^{2+}]_i$ [13].

Prior to exposure to OFF, cells were washed with PBS, incubated with 10 μM Fura-2 AM in α-MEM and 2% FBS for 30 min at 37 °C, and then washed again with PBS [1]. Following Fura-2 loading, the slides were mounted in the flow chamber, and then fixed to the stage of a Nikon TE300 epi-fluorescent microscope (Nikon Inc., Melville, NY). The setup was left undisturbed for 30 min, during which the cells were perfused with α-MEM media and 2% FBS at 0.05 ml/min. Cells were then subjected to 3 min of OFF at 1 Hz with peak shear stress of 20 dynes/cm^2. Ratio images were acquired every 2 s for 3 min prior to flow, for the duration of the OFF, and for 3 min after the flow was terminated. Ratio images were recorded and analyzed using image analysis software (Metafluor; Universal Imaging, West Chester, PA). Fura-2 ratio values were converted to $[Ca^{2+}]_i$ levels with a calibration curve derived from a series of standard Ca^{2+} solutions provided by the manufacturer.

Cell proliferation

Cell proliferation was assessed by examining the incorporation of BrdU, a thymidine analog (Absolute-S SBIP Cell Proliferation Assay Kit). For the BrdU, gene expression, and ALP activity experiments, cells were subjected to OFF for 2 h at 1 Hz with peak shear stress of 10 dynes/cm^2 while perfused with fresh complete growth media at 0.05 ml/min [44]. After OFF, cells were incubated in fresh complete growth media. The cells in the control group were cultured under identical conditions but received no flow. Cells were collected 24 h after OFF and incubated with BrdU for 30 min. Then the cells were photolysed and labeled. BrdU was detected by an anti-BrdU antibody using standard immunohistochemical techniques. The fraction of BrdU-positive cells was counted using the epi-fluorescent microscope.

Gene expression

Gene expression was quantified using real time reverse transcriptase polymerase chain reaction (real time RT-PCR) [44]. Four osteoblast marker genes were studied: Cbfa1, OP, OSTC, and Col I. Cells were cultured and subjected to OFF as described above. Twenty-four hours after OFF, total RNA was isolated (RNeasy Mini kit). Cells were lysed, homogenized, and bound to a silica-gel-based membrane to obtain high quality total RNA. Real time RT-PCR was performed at the Penn State Nucleic Acid Facility using the ABI PRISM 7700 Sequence Detection system (Applied Biosystems, Foster City, CA). For each sample, mRNA levels for each gene were normalized to the housekeeping 18S rRNA levels and also normalized to the negative control.

The following primers and probes were used: **Cbfa1**: the forward primer was 5′FAM-TGC TTC ATT CGC CTC ACA AA-TAMRA-3′; the reverse primer was 5′FAM-TGC TGT CCT CCT CGA GAA AGT T-TAMRA-3′; and the probe was 5′-AAC CAC AGA ACC ACA AGT GCG GTG C-3′. **OP**: the forward primer was 5′FAM-TTG CAG CCT TCT CAG CCA A-TAMRA-3′; the reverse primer was 5′FAM-CAA AAG CAA ATC ACT GCA ATT CTC-TAMRA-3′; and the probe was 5′-CGC GGA CCA AGG AAA ACT CAC TAC CA-3′. **Col I**: the forward primer was 5′FAM-CGC ACG GCC AAG AGG A-TAMRA-3′; the reverse primer was 5′FAM-ACG CAG GTG ATT GGT GGG-TAMRA-3′; and the probe was 5′-CAA GTC GAG GGC CAA GAC GAA GAC A-3′. **OSTC**: the forward primer was 5′FAM-GCA GGT GCG AAG CCC A-TAMRA-3′; the reverse primer was 5′FAM-ACC CTA GAC CGG GCC GT-TAMRA-3′; and the probe was 5′-TTT CAG GAG GCC TAT CGG CGC TTC-3′.

Alkaline phosphatase

ALP activity was measured by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol (Sigma 104 kit) [5]. Cells were cultured and subjected to OFF as described above. ALP activity was assayed 3 days after OFF, at 6–8 days after plating. Cells were lysed using 0.05% triton and freeze–thawed twice, then the substrate was added. The end-product, *p*-nitrophenol, was measured by absorption at 415 nm and converted to Sigma units using a *p*-nitrophenol standard absorption curve. In parallel, total protein concentration from each slide was measured to normalize ALP activity by cell count (Pierce BCA kit).

Data analysis

Calcium imaging data were analyzed using the Rainflow cycle counting numerical procedure. This method identifies oscillations in the time history data and is used to quantify $[Ca^{2+}]_i$ responses from background noise [17]. Calcium imaging data were expressed as the fractions of cells responding ± SE and mean changes in $[Ca^{2+}]_i$ ± SE. Statistical analyses using one-way ANOVA and Fisher's Protected Least Significant Difference were utilized to detect significant differences between groups; $p < 0.05$ was considered significant. BrdU incorporation, gene expression, and ALP activity data were expressed as means ± SE. Statistical analyses were performed using two-way ANOVA followed by the Dunnett's post hoc test using StatView software (Cary, NC); $p < 0.05$ was again considered significant.

Results

Calcium imaging

OFF triggered potent $[Ca^{2+}]_i$ responses in MSCs. Two parameters were used to quantify the responsiveness: the fraction of cells that responded to OFF and the mean change in $[Ca^{2+}]_i$ amplitude. A responding cell was defined as one exhibiting increased intracellular calcium levels in response to flow of more than four times its maximum pre-flow variability [17]. Three slides were observed for a total of 60 cells. Without OFF, the $[Ca^{2+}]_i$ fluctuations were small and very few cells exhibited response behavior (Fig. 1). When OFF was applied, one or more dramatic and transient increases in $[Ca^{2+}]_i$ were observed in most cells. The first $[Ca^{2+}]_i$ responses occurred roughly 10 s after the start of OFF. The fraction of cells that responded to OFF (89 ± 5.4% of cells) was significantly greater than the pre-flow control period (4.6 ± 2.5%, $p < 0.0001$) (Fig. 2A). When the magnitudes of the response were compared (Fig. 2B), the mean change in $[Ca^{2+}]_i$ (of the responding cells) was significantly higher during OFF (224 nM ± 129, $p < 0.001$) than during the pre-flow control period (23 nM ± 27, $p < 0.001$).

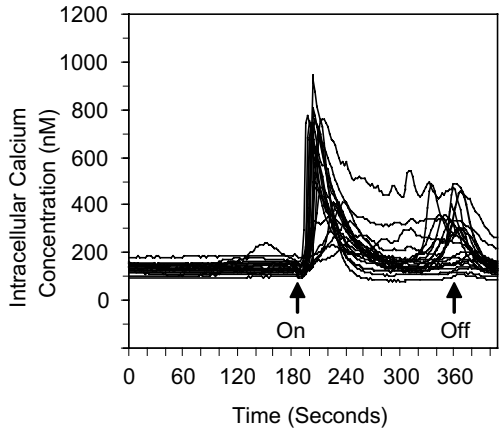

Fig. 1. Examples of Ca^{2+} time history traces where each trace represents $[Ca^{2+}]_i$ levels in one cell. MSCs responded to OFF with dramatic transient increases in $[Ca^{2+}]_i$. Arrows mark time points where OFF was turned on and off. The first $[Ca^{2+}]_i$ responses occurred roughly 10 s after the start of OFF.

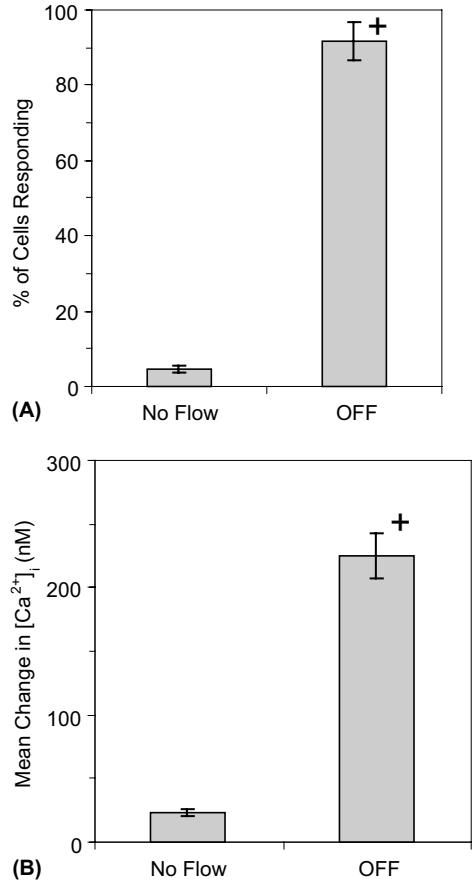

Fig. 2. (A) Percent of cells responding to OFF: a cell's responsiveness was quantified as exhibiting increased $[Ca^{2+}]_i$ levels in response to flow of more than four times its pre-flow variability. The control group is the percentage of cells that responded during the pre-flow period. + indicates a significant difference from control ($p < 0.0001$). (B) Mean change in magnitude of $[Ca^{2+}]_i$ increase in responding cells: + indicates significance difference between OFF and control ($p < 0.001$).

Cell proliferation

OFF significantly increased the proliferation of MSCs (Fig. 3). MSCs subjected to OFF had significantly higher fractions of BrdU incorporation than MSCs not subjected to OFF (57% increase; $n = 15$ for cells exposed to OFF, $n = 25$ for cells not exposed to OFF).

Gene expression

OFF significantly altered the gene expression of MSCs (Fig. 4). Six experiments were conducted with total sample size (n) of 17–22 slides for each experimental group. Cells subjected to OFF had significantly higher OP (65% increase) and OSTC (44% increase) mRNA levels (normalized to 18S rRNA and normalized

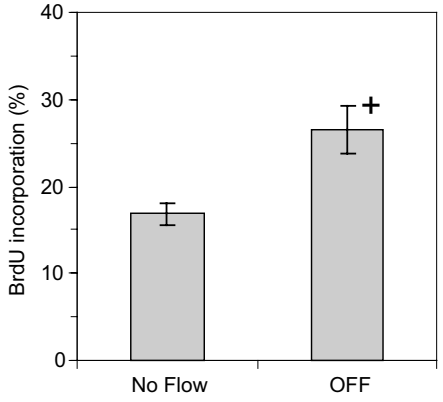

Fig. 3. OFF increased the rate of proliferation of MSCs. Cells exhibited increased BrdU incorporation 24 h after OFF (57% increase). + indicates significant difference between MSCs exposed to OFF ($n = 15$) and MSCs not exposed to OFF ($n = 25$; $p < 0.05$).

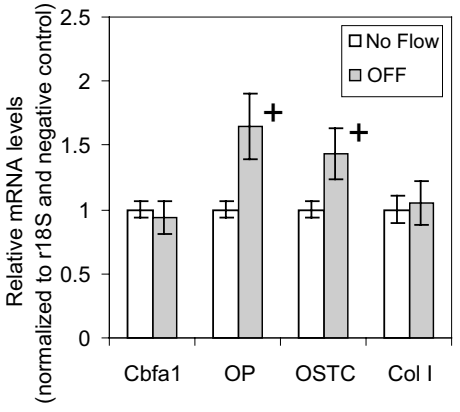

Fig. 4. Effects of OFF on relative mRNA levels for MSCs: mRNA isolation 24 h after OFF. mRNA levels normalized to 18S rRNA and normalize to the negative control. + indicates significant difference between MSCs subjected to OFF and MSCs not exposed to OFF: OP (65% increase) and OSTC (44% increase) ($p < 0.05$; $n = 17$–22).

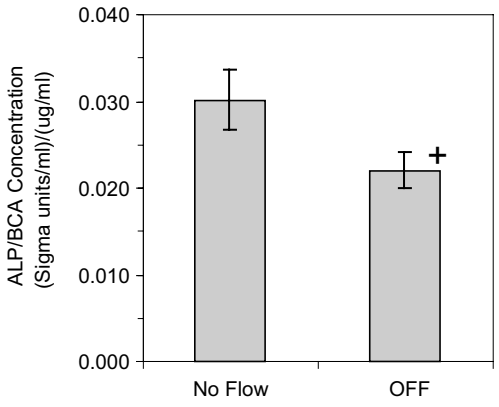

Fig. 5. OFF decreased alkaline phosphatase activity levels of MSCs (27% decrease). ALP activity assayed 3 days after OFF. + indicates significant difference between MSCs subjected to OFF and MSCs not subjected to OFF ($p < 0.05$, $n = 9$).

to the negative control). The Cbfa1 and Col I mRNA levels were not significantly different.

Alkaline phosphatase

OFF significantly decreased ALP activity in MSCs cultured in osteogenic media. ALP activity was measured by the accumulation of the reaction product *p*-nitrophenol after 5, 10, 20, and 30 min of incubation. For all samples, *p*-nitrophenol levels increased over time and were not saturated at 30 min (Fig. 5). MSCs subjected to OFF had significantly lower ALP activity than MSCs not subjected to flow (27% decrease, $n = 9$ slides).

Discussion

The results of this study show that MSCs respond to OFF with transient increases in intracellular Ca^{2+}, increased proliferation rate, upregulation in osteoblastic gene expression, and decreased ALP activity. The intracellular Ca^{2+} response to OFF in MSCs was similar to previous studies using osteoblastic cells, such as MC3T3-E1 and hFOB, as well as the osteocytic line MLO-Y4 [8]. In both cell types, usually a single dramatic increase in $[Ca^{2+}]_i$ occurred shortly after the onset of flow [44,45]. In osteoblastic cells, You et al. [44] found that OFF resulted in increased OP levels; however, the effects were completely abolished when the Ca^{2+} response was blocked. This may also be the case for MSCs. However, to determine whether MSCs share similar $[Ca^{2+}]_i$ transduction pathways to osteoblastic cells, additional experiments that include an array of pharmacological blockers to characterize the specific biochemical signaling pathways involved are needed.

This study also demonstrated that OFF can increase the rate of proliferation of MSCs. The increase in proliferation as a result of mechanical stimulus is consistent with in vivo studies where mechanical unloading led to a decrease in cell proliferation. Zhang et al. [46] found that tail suspension in rats resulted in a decreased MSC population, decreased proliferation of ALP positive cells, decreased IGF-II production, and OP mRNA levels. Similarly, using BrdU immunohistochemistry, Barou et al. [2] found decreased proliferation of MSCs in tail suspended rats. There is an important link with long-term homeostatic bone remodeling because osteoblasts are constantly renewed by proliferating osteoprogenitors.

Our gene results suggest that OFF affects the osteogenic differentiation of MSCs. Cbfa1, a mammalian homolog of Drosophilia Runt protein, is a critical regulator of osteoblast differentiation. Genetically altered Cbfa1-deficient mice have a skeleton devoid of osteoblasts and bone matrix [20]. Our finding that OFF upregulated OP and OSTC but did not upregulate Cbfa1 is a pattern that has been reported in gene expression of differentiating osteoblastic cells [40], suggesting that OFF may act downstream of Cbfa1 to stimulate OP and OSTC. For example, osterix (Osx) is a transcription factor that regulates osteoblastic differentiation downstream of Cbfa1. Osx-deficient mice lack osteoblasts and bone matrix even though Cbfa1 is expressed normally [27].

Our results showed that OFF upregulated OP and OSTC, which suggests increased osteoblastic differentiation of MSCs. OP and OSTC are indicators of osteoblastic differentiation, whose functions in the bone matrix continue to be intensely studied. In bone, OP has been found to regulate bone cell adhesion, osteoclast function, and matrix mineralization. Intermittent mechanical strain has been found to upregulate OP levels [14]. However, skeletal changes were not found in OP-null mice, which indicates that other matrix molecules may substitute for OP [12]. OSTC, a bone specific matrix protein, has increased expression during mineralization and is regulated by Cbfa1 and vitamin D_3 [26,34]. Mizuno and Kuboki [26] found that OP and OSTC were upregulated in MSCs after mineralization (3 weeks culture time in a type I collagen matrix).

OFF did not significantly alter Col I mRNA levels. Control cells expressed Col I mRNA at a high basal level, and as a result, the effect of OFF on Col I mRNA levels may not have been strong enough to overcome the high basal expression of Col I mRNA. This finding is consistent with Frank et al. [10], who found that MSCs cultured in osteogenic media did not show significant changes in Col I over a 20 day culture period.

Increased ALP activity has been used in many studies as an indicator of osteoblast differentiation [5,9,18, 19,26,30,36]. However, we found that OFF actually reduced ALP activity in MSCs 3 days after stimulation. This was unexpected since we also found increased OP

and OSTC mRNA levels, suggesting increased MSC differentiation 24 h after exposure to OFF. However, there are reports in the literature of osteoblastic subclones that exhibit mineralization without increased ALP mRNA activity [40], and ALP has been reported to be biphasic in MSCs with a decrease in activity prior to mineralization [18]. Thus, it appears that ALP may be regulated independently from other markers of osteogenic differentiation. Furthermore, in prior studies of physical signals applied to bone cells, Stanford et al. [35] found depressed ALP activity as a response to mechanical deformation, and Hillsley and Frangos [15] found that pulsatile fluid flow down-regulated ALP activity. Thus, although our finding of reduced ALP activity with mechanical stimulation was unexpected, it is consistent with prior investigations of this type and may be a consequence of an incomplete understanding of the specific roles of ALP in differentiation.

A somewhat related issue is raised by our finding that OFF was found to both increase the proliferation of MSCs and increase markers of osteogenic markers of differentiation, with the notable exception of ALP activity. The traditional view resulting from the study of primary bone cell culture is that decreased proliferation and arrest of the cell cycle precedes osteogenic differentiation [9,36]. These seemingly paradoxical findings can be reconciled since our heterogeneous MSC population includes progenitor cells at various stages of differentiation. It may be that OFF results in increased proliferation in a subpopulation of early progenitor cells while simultaneously resulting in increased differentiation of another fraction of osteogenic precursors. Indeed simultaneous increases in proliferation and osteogenic differentiation of human MSCs have been reported in response to biochemical stimulation [18]. This issue could be addressed by assaying individual cells for ALP activity and simultaneously conducting a cell cycle analysis of synchronized cultures.

Insights into the MSC response to mechanical signals will enable better understanding and treatment of bone loss diseases such as osteoporosis and disuse bone loss. While previous studies have focused on osteoblasts, osteocytes, and osteoclasts, MSCs' response may contribute directly to bone's response to mechanical loading. In addition, MSCs offer novel treatment options in tissue engineering applications. Marrow cells can be harvested from an individual, then culture expanded in tissue scaffolds and subsequently re-implanted. In addition to biochemical signals, future tissue engineers may be able to manipulate the mechanical environment to regulate MSC proliferation and differentiation.

In summary, this study found OFF to be a potent regulator of MSC proliferation and differentiation. OFF triggered intracellular Ca^{2+} mobilization, where Ca^{2+} is an important second messenger in the mechanotransduction pathway. OFF also significantly increased MSC proliferation, thereby increasing the number of potential osteoprogenitors. In addition, OFF upregulated osteoblastic gene expression of OP and OSTC and lowered the ALP activity of MSCs.

Acknowledgements

Thanks to Dr. Deborah Grove for her technical assistance with real time RT-PCR analysis. Thanks to Dr. Jun You, Donielle Newell, and Kyung Park for their technical expertise and assistance. This work was supported by NIH Grant AR 45989, NASA Grant NAG2-1601, and US Army Medical Research Award DAMD 17-98-1-8509.

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.
[2] Barou O, Palle S, Vico L, Alexandre C, Lafage-Proust MH. Hindlimb unloading in rat decreases preosteoblast proliferation assessed in vivo with BrdU incorporation. Am J Physiol 1998; 274:E108–14.
[3] Chow JW, Wilson AJ, Chambers TJ, Fox SW. Mechanical loading stimulates bone formation by reactivation of bone lining cells in 13-week-old rats. J Bone Miner Res 1998;13:1760–7.
[4] Dehority W, Halloran BP, Bikle DD, Curren T, Kostenuik PJ, Wronski TJ, et al. Bone and hormonal changes induced by skeletal unloading in the mature male rat. Am J Physiol 1999;276: E62–9.
[5] Donahue HJ, Li Z, Zhou Z, Yellowley CE. Differentiation of human fetal osteoblastic cells and gap junctional intercellular communication. Am J Physiol Cell Physiol 2000;278:C315–22.
[6] 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.
[7] Ducy P, Karsenty G. Skeletal Gla proteins: gene structure, regulation of expression and function. In: Bilezikian JP, editor. Principles of bone biology. San Diego: Academic Press; 1996. p. 183–95.
[8] Edlich M, Yellowley CE, Jacobs CR, Donahue HJ. Effect of oscillating flow on osteocytic cells is dependent on gap junctional intercellular communication. Trans Orthop Res Soc 2000:323.
[9] Fedarko NS, Bianco P, Vetter U, Robey PG. Human bone cell enzyme expression and cellular heterogeneity: correlation of alkaline phosphatase enzyme activity with cell cycle. J Cell Physiol 1990;144:115–21.
[10] Frank O, Heim M, Jakob M, Barbero A, Schafer D, Bendik I, et al. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem 2002;85:737–46.
[11] Friedenstein AJ, Piatetzky II S, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966;16:381–90.
[12] Giachelli CM, Steitz S. Osteopontin: a versatile regulator of inflammation and biomineralization. Matrix Biol 2000;19:615–22.
[13] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.

[14] Harter LV, Hruska KA, Duncan RL. Human osteoblast-like cells respond to mechanical strain with increased bone matrix protein production independent of hormonal regulation. Endocrinology 1995;136:528–35.
[15] Hillsley MV, Frangos JA. Alkaline phosphatase in osteoblasts is down-regulated by pulsatile fluid flow. Calcif Tissue Int 1997;60:48–53.
[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.
[17] Jacobs CR, Yellowley CE, Nelson DV, Donahue HJ. Analysis of time-varying biological data using rainflow cycle counting. Comput Methods Biomech Biomed Eng 2000;3:31–40.
[18] Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 1997;64:295–312.
[19] Keila S, Pitaru S, Grosskopf A, Weinreb M. Bone marrow from mechanically unloaded rat bones expresses reduced osteogenic capacity in vitro. J Bone Miner Res 1994;9:321–7.
[20] Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755–64.
[21] Kurokouchi K, Jacobs CR, Donahue HJ. Oscillating fluid flow inhibits TNF-alpha-induced NF-kappa B activation via an Ikappa B kinase pathway in osteoblast-like UMR106 cells. J Biol Chem 2001;276:13499–504.
[22] Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 1997;12:1335–47.
[23] Lafage-Proust MH, Collet P, Dubost JM, Laroche N, Alexandre C, Vico L. Space-related bone mineral redistribution and lack of bone mass recovery after reambulation in young rats. Am J Physiol 1998;274:R324–34.
[24] Machwate M, Zerath E, Holy X, Hott M, Modrowski D, Malouvier A, et al. Skeletal unloading in rat decreases proliferation of rat bone and marrow-derived osteoblastic cells. Am J Physiol 1993;264:E790–9.
[25] McAllister TN, Du T, Frangos JA. Fluid shear stress stimulates prostaglandin and nitric oxide release in bone marrow-derived preosteoclast-like cells. Biochem Biophys Res Commun 2000; 270:643–8.
[26] Mizuno M, Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. J Biochem (Tokyo) 2001;129:133–8.
[27] Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17–29.
[28] Pavlin D, Dove SB, Zadro R, Gluhak-Heinrich J. Mechanical loading stimulates differentiation of periodontal osteoblasts in a mouse osteoinduction model: effect on type I collagen and alkaline phosphatase genes. Calcif Tissue Int 2000;67:163–72.
[29] Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci USA 1995;92:4857–61.
[30] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.
[31] Plant A, Tobias JH. Characterisation of the temporal sequence of osteoblast gene expression during estrogen-induced osteogenesis in female mice. J Cell Biochem 2001;82:683–91.
[32] Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–4.
[33] Saunders MM, You J, Trosko JE, Yamasaki H, Li Z, Donahue HJ, et al. Gap junctions and fluid flow response in MC3T3-E1 cells. Am J Physiol Cell Physiol 2001;281:C1917–25.
[34] Sierra J, Villagra A, Paredes R, Cruzat F, Gutierrez S, Javed A, et al. Regulation of the bone-specific osteocalcin gene by p300 requires Runx2/Cbfa1 and the Vitamin D3 receptor but not p300 Intrinsic Histone Acetyltransferase activity. Mol Cell Biol 2003;23:3339–51.
[35] Stanford CM, Morcuende JA, Brand RA. Proliferative and phenotypic responses of bone-like cells to mechanical deformation. J Orthop Res 1995;13:664–70.
[36] Stein GS, Lian JB, Owen TA. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. Faseb J 1990;4:3111–23.
[37] Thomas GP, el Haj AJ. Bone marrow stromal cells are load responsive in vitro. Calcif Tissue Int 1996;58:101–8.
[38] Turner CH, Owan I, Alvey T, Hulman J, Hock JM. Recruitment and proliferative responses of osteoblasts after mechanical loading in vivo determined using sustained-release bromodeoxyuridine. Bone 1998;22:463–9.
[39] van der Meulen MC, Morey-Holton ER, Carter DR. Hindlimb suspension diminishes femoral cross-sectional growth in the rat. J Orthop Res 1995;13:700–7.
[40] Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res 1999;14:893–903.
[41] Weinbaum S, Cowin SC, Zeng Y. A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. J Biomech 1994;27:339–60.
[42] Wronski TJ, Morey-Holton ER, Doty SB, Maese AC, Walsh CC. Histomorphometric analysis of rat skeleton following spaceflight. Am J Physiol 1987;252:R252–5.
[43] Yang X, Karsenty G. Transcription factors in bone: developmental and pathological aspects. Trends Mol Med 2002;8:340–5.
[44] You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, et al. Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem 2001;276:13365–71.
[45] 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.
[46] Zhang R, Supowit SC, Klein GL, Lu Z, Christensen MD, Lozano R, et al. Rat tail suspension reduces messenger RNA level for growth factors and osteopontin and decreases the osteoblastic differentiation of bone marrow stromal cells. J Bone Miner Res 1995;10:415–23.