Substrate Deformation Levels Associated With Routine Physical Activity Are Less Stimulatory to Bone Cells Relative to Loading-Induced Oscillatory Fluid Flow

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Although it is well accepted that bone tissue metabolism is regulated by external mechanical loads, it remains unclear to what load-induced physical signals bone cells respond. In this study, a novel computer-controlled stretch device and parallel plate flow chamber were employed to investigate cytosolic calcium ($Ca^{2+}{}_i$) mobilization in response to a range of dynamic substrate strain levels (0.1–10 percent, 1 Hz) and oscillating fluid flow (2 N/m^2, 1 Hz). In addition, we quantified the effect of dynamic substrate strain and oscillating fluid flow on the expression of mRNA for the bone matrix protein osteopontin (OPN). Our data demonstrate that continuum strain levels observed for routine physical activities (<0.5 percent) do not induce $Ca^{2+}{}_i$ responses in osteoblastic cells in vitro. However, there was a significant increase in the number of responding cells at larger strain levels. Moreover, we found no change in osteopontin mRNA level in response to 0.5 percent strain at 1 Hz. In contrast, oscillating fluid flow predicted to occur in the lacunar–canalicular system due to routine physical activities (2 N/m^2, 1 Hz) caused significant increases in both $Ca^{2+}{}_i$ and OPN mRNA. These data suggest that, relative to fluid flow, substrate deformation may play less of a role in bone cell mechanotransduction associated with bone adaptation to routine loads. [S0148-0731(00)01204-8]

Introduction

It is well known that mechanical force is an important factor affecting bone adaption and formation. Removal of mechanical stimulation causes reduced bone matrix protein production, mineral content, and bone formation as well as an increase in bone resorption [1]. Conversely, increased mechanical loading can increase bone formation and decrease bone resorption [2]. Therefore, bone is capable of altering its external shape and internal structure to support the load bearing demands placed upon it efficiently. However, the mechanism by which bone cells sense and respond to their physical environment, including mechanical loading, is still poorly understood. This study is focused on the cellular level physical signals induced by external mechanical loading to which bone cells might be responding.

A number of potential physical signals have been investigated in terms of their ability to regulate bone cell metabolism with *in vitro* culture systems. However, the majority of these experiments have been criticized for either applying stimulus levels much in excess of those occurring in response to the routine bone loads or for employing mechanical stimulation systems that induce unintended physical signals. For example, bone cells are known to respond to electric fields; however, the levels of endogenously occurring electric fields have only a modest effect [3]. Another possibility is that bone cells directly sense the deformation of the substrate to which they are attached. Indeed, substrate deformation has been shown to influence DNA synthesis, second messenger production (cyclic AMP), release of paracrine factors (PGE_2), the activity of enzymes important to mineralization (alkaline phosphatase), and both collagenous and non-collagenous matrix protein synthesis [4–6]. However, the flexible membranes utilized as substrates in these studies exposed the cells to deformations of between 5-fold and 125-fold greater than observed to occur in bone during routine physical activity. For example, in humans during vigorous exercise, surface strains remain below 0.2 percent [7]. To apply smaller deformations, some investigators have utilized systems based on bending of rigid substrates such as glass slides [8–11]. Unfortunately this approach exposes the cells to significant fluid shear stress due to the lateral motion of the slide through the bathing media. This unintended fluid flow is complex and potentially turbulent in nature. Moreover, an appropriate control is problematic, since rocking or tilting of a control slide is unlikely to result in the same cellular fluid environment as the bending slide [9,11]. Owan et al. [10] were able to distinguish the effects of strain from the effects of fluid flow by varying slide thickness. They concluded that, with respect to osteopontin (OPN) expression, fluid flow was the predominant physical stimulus and that there was no response to strain levels below 8 percent. The approach taken in this study was to utilize a system designed to apply substrate deformation over a range from 0.1–10 percent without exposing the cells to fluid flow.

An alternative to the hypothesis that bone cells respond directly to deformation is that they sense loading-induced flow of fluid through the lacunar–canalicular network [12,13]. To date it has proven impossible to quantify lacunar–canalicular flow magnitudes. However, Weinbaum et al. [14] predicted that the wall shear stress levels experienced by bone cells *in vivo* range from 0.8–3 N/m^2 on the basis of a theoretical model validated with respect to the streaming potentials [13]. Smalt et al. [15] utilized a system capable of exposing bone cells to levels of substrate strain comparable to those experienced by bone cells *in vivo* during routine physical activities without induced fluid flow. They found no increase in NO or PGE_2 production in response to strain (0.05–

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0.5 percent). In contrast, exposure to fluid flow induced both PGE_2 and NO production in osteoblastic cells. However, these studies did not address the very early intracellular signaling event of intracellular calcium ($Ca^{2+}{}_i$) mobilization, which may be sensitive to substrate strain without affecting PGE_2 or NO production. Also, they did not employ a dynamic or oscillatory flow profile, which would be the predominant type of flow *in vivo* due to the dynamic nature of routine physical activities [16] and has been demonstrated to mobilize $Ca^{2+}{}_i$ *in vitro* [17].

$Ca^{2+}{}_i$ is an early response second messenger that plays a role in a number of metabolic pathways, and is typically observed to increase dramatically within seconds of stimulation. As a second messenger, $Ca^{2+}{}_i$ transduces extracellular changes (i.e., first messenger) to the cell interior and potentially to the genome, and is important in the regulation of cellular metabolism. $Ca^{2+}{}_i$ mobilization in response to steady fluid flow in bone cells has been studied [18], but not in response to substrate strain at levels known to occur during routine physical activities. We have taken an approach that allows us to distinguish the effects of dynamic substrate strain from those of fluid flow by applying precisely controlled, varied dynamic substrate strain levels, without induced fluid flow.

In addition to $Ca^{2+}{}_i$, we chose also to quantify mRNA levels of the osteopontin (OPN) gene in response to substrate deformation and fluid flow. OPN is characterized as one of the predominant noncollagenous proteins that are accumulated in the extracellular matrix of bone in a wide variety of vertebrates [19,20]. OPN is also believed to be an important factor associated with bone remodeling caused by mechanical stress *in vivo* [21]. In several recent studies expression levels of mRNA for OPN were shown to be modulated in response to mechanical stimulation of bone cells *in vitro* [6,22,23]. However, these data were obtained with applied strains in excess of those expected to occur during routine physical activities.

In this study we exposed bone cells to controlled levels of dynamic substrate strain ranging from 0.1–10 percent at 1 Hz in the absence of induced fluid flow and also to oscillating dynamic fluid flow expected to occur due to routine physical activities in the absence of substrate strain. In order to ensure that our findings are not an artifact of our cell culture model, we employed a variety of bone cell types including human osteoblastic cells (hFOB), primary cultures of rat osteoblasts (ROB), and osteocytic cells (MLO-Y4). In terms of biological outcomes, we quantified real-time changes in $Ca^{2+}{}_i$ and steady-state mRNA levels for the OPN gene. Our experimental design addresses several significant unstudied questions including: the dose response relationship between dynamic substrate strain and $Ca^{2+}{}_i$; whether $Ca^{2+}{}_i$ is affected by dynamic substrate strain levels expected to occur during routine physical activities; how this response compares with the response to levels of dynamic lacunar–canalicular fluid flow expected to occur during routine physical activities; and how these two physical signals independently affect OPN mRNA levels.

Materials and Methods

Cell Culture. Human fetal conditionally immortalized osteoblastic cells (hFOB 1.19) [24] were employed in this study. These cells were transfected with a temperature-sensitive promoter linked to the SV40 gene such that the gene is expressed at 37°C and expression is inhibited when the cells are cultured at 39.5°C. hFOB cells were grown in Dulbecco's modified Eagle medium (DMEM) with nutrient mixture F-12 (DMEM/F12, Gibco, Gaithersburg, MD) supplemented with 10 percent fetal bovine serum (FBS) and 1 percent penicillin and streptomycin at 37°C. Before experiments, hFOB cells were transferred into a 39.5°C incubator for 24 hours to express characteristics of mature osteoblasts [24]. To confirm that our dynamic substrate strain responses were not an artifact of the behavior of the cell line, we also utilized primary cultures of rat subperiosteal osteoblastic cells (ROB) and osteocytic (MLO-Y4) cells. ROB cells were isolated from long bones of four-month-old male Fischer-344 rats, as previously described [25]. We have demonstrated that cells isolated in this manner express characteristics of the osteoblast phenotype. ROB cells were cultured in DMEM containing 20 percent FBS and 1 percent penicillin and streptomycin. Osteocyte-like cells (MLO-Y4) [26] provided by Dr. Lynda Bonewald (University of Texas Health Science Center, San Antonio) were also tested for dynamic substrate strain responses. MLO-Y4 cells were cultured in alpha modified essential medium (αMEM) supplemented with 5 percent FBS, 5 percent calf serum (CS) and 1 percent penicillin and streptomycin. In the stretch experiments ROB and hFOB cells were grown on fibronectin-coated silicone membranes (Flexcell International, Hillsborough, NC) to enhance adhesion. MLO-Y4 cells were cultured on type I collagen (Flexcell International, Hillsborough, NC), which is required to maintain the osteocyte-like phenotype [26]. For the flow experiments, bone cells were cultured on quartz glass slides (76 mm×26 mm×1.6 mm) for calcium imaging and on normal glass slides (75 mm×38 mm×1.0 mm) for osteopontin studies.

Dynamic Substrate Stretch Device. Our dynamic substrate deformation apparatus consisted of a coated silicone membrane and a computer-controlled ZETA 6104 motor-driven micrometer (Parker Hannifin Corp., Rohner Park, CA). One end of the membrane was fixed to the microscope stage and the other end was connected to a micrometer (Fig. 1). The motor can be accurately positioned within 1 μm under computer control to apply the desired dynamic strain. Dynamic strains consisted of a triangle waveform ranging from zero to maximal tensile strain at 1 Hz. Strains in the substrate were verified by tracking optical markers to confirm that the substrate was accurately deformed. Cells were cultured on the precoated membranes within a plastic ring filled with medium (Tyrode's solution with 2 percent FBS) that minimized the fluid flow during dynamic stretch. The whole dynamic stretch device was placed on the stage of a Nikon Diaphot inverted microscope equipped for epifluorescence and computer image acquisition.

Fluid flow levels induced by our dynamic stretch apparatus were extremely low. For instance, 1 percent strain in our apparatus only creates 0.0001 N/m^2 average wall shear stress [27] and our data suggest that there is no $Ca^{2+}{}_i$ response for such low levels of shear stress. Likewise, 0.68 mm microscope slides were utilized in the flow experiments to eliminate substrate deformation.

Fluid Flow Device. A parallel plate flow chamber was used to apply fluid flow [17]. The design was modified from Frangos et al. [28] to accept the UV transparent quartz glass microscope slides required for fluorescent imaging. The flow rate was quantified with an ultrasonic flowmeter (Transonic Systems Inc., Ithaca, NY). Details of the chamber and the computer-controlled dynamic

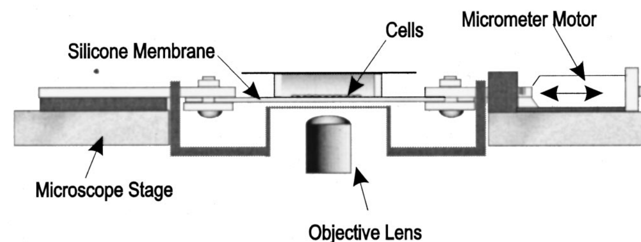

Fig. 1 Schematic of the substrate stretch device consisting of a silicone membrane and a computer motor-driven micrometer. One end of the membrane was fixed to the microscope stage and the other end was connected to the micrometer. Cells were cultured on the precoated membrane and a plastic ring filled with medium was placed on the membrane.

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flow delivery system are given in Jacobs et al. [17]. For calcium studies, the rectangular fluid volume measured 38 mm×10 mm ×0.28 mm. A larger flow chamber with a rectangular fluid volume of 56 mm×24 mm×0.28 mm was employed for OPN studies to increase the mRNA yield.

Calcium Imaging. Intracellular calcium ion concentration ($[Ca^{2+}]_i$) was quantified with the fluorescent dye fura-2. Fura-2 exhibits a shift in absorption when bound to calcium such that emission intensity when illuminated with ultraviolet light at a wavelength of 340 nm increases with calcium concentration, and decreases with calcium concentration when illuminated at 380 nm.

Preconfluent (80 percent) cells were washed with Tyrode's solution at 37°C (39.5°C for hFOB), which contained 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM N-2-hydroxyethylipiperazine-N-2-ethanesulphonic acid and 10 mM glucose, titrated to a final pH of 7.4 with 4 mM NaOH. Cells were then incubated with 1 μM fura-2-acetoxymethyl ester (Molecular Probes, Eugene, OR) solution for 30 min at 37°C (39.5°C for hFOB). The cells were then washed again with fresh Tyrode's solution prior to experiments.

Cell ensembles were illuminated at wavelengths of 340 and 380 nm in turn. Emitted light was passed through a 510 nm interference filter and detected with an ICCD camera (International LTD., Sterling, VA). Images were recorded at a rate of one every second and analyzed using image analysis software (Metafluor; Universal Imaging, West Chester, PA). Calibration ratios were obtained using fura-2 in buffered calcium standards supplied by the manufacturer (Molecular Probes, Inc., Eugene, OR). Basal $[Ca^{2+}]_i$ was sampled for 0.5 min followed by cyclic mechanical stretch. We first induced dynamic strains of 0.1 percent for 0.5 min followed by a 3 min rest period then 1 percent strain, rest, 5 percent, rest, 10 percent, and then rest (Fig. 2). The dynamic stretch was applied as a triangle wave at a frequency of 1 Hz. Also note that the corresponding strain rates were 0.2 percent/s, 2 percent/s, 10 percent/s, and 20 percent/s, respectively. $[Ca^{2+}]_i$ images were collected during the no-stretch rest period only. To negate any possible cumulative effect on the $[Ca^{2+}]_i$ responses of the different dynamic strains, we also applied the strain in order of decreasing magnitude (0.5 min no stretch period followed by a 3 min rest period then 10 percent strain, rest, 5 percent, rest, 1 percent, rest, 0.1 percent and then rest). For flow experiments, basal $[Ca^{2+}]_i$ was sampled for 0.5 min followed by fluid flow onset.

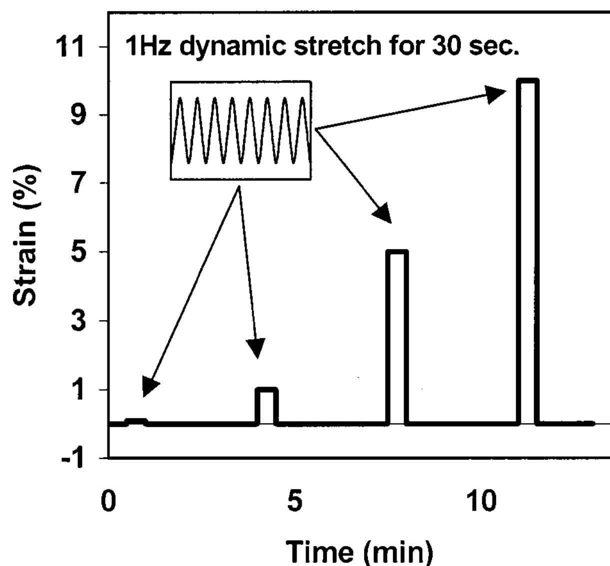

Fig. 2 Membrane stretch pattern. We first induced dynamic strains of 0.1 percent for 0.5 min followed by a 3 min rest period then 1 percent strain, rest, 5 percent, rest, 10 percent, and then rest. The order of strain levels was also reversed. The strain waveform was a triangle wave and the frequency for all strain experiments was 1 Hz.

Osteopontin mRNA Analysis. Quantitative real-time reverse transcription PCR (QRT RT-PCR) was employed to quantify steady-state osteopontin mRNA levels. This technique is based on the detection of a fluorescent signal produced by an OPN-specific oligonucleotide probe during PCR amplification. The RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) was used to extract total RNA. Briefly, cells were washed with PBS, lysed, and homogenized using the QIAshredder mini column (Qiagen, Inc., Valencia, CA). After binding to the RNeasy column, total RNA was eluted with RNase-free water and collected. The osteopontin mRNA level was determined by using quantitative real time RT-PCR (Prism 7700 Sequence Detector, Applied Biosystems, Frost City, CA). The fluorogenic oligonucleotide probe for human osteopontin was 5'-CGC CGA CCA AGG AAA ACT CAC TAC CA-3' (Synthetic Genetics, San Diego, CA). The forward and reverse PCR primers were 5'-TTG CAG CCT TCT CAG CCA A-3', and 5'-CAA AAG CAA ATC ACT GCA ATT CTC-3', respectively [29]. Relative changes in the levels of OPN mRNA and 18S rRNA were quantified 72 hours after mechanical stimulation.

Data Analysis. We used a numerical procedure from mechanical analysis, known as Rainflow cycle counting, to identify calcium oscillations [17,30]. Briefly, this technique identifies complete cycles or oscillations in the time history data even when they are superimposed upon each other, and therefore can be used to distinguish and quantify $[Ca^{2+}]_i$ responses from background noise. We defined a response as an oscillation in $[Ca^{2+}]_i$ of 20 nm or greater. Base line $[Ca^{2+}]_i$ data were recorded for 0.5 min followed by application of desired dynamic substrate strain or fluid flow.

Data were expressed as mean ±SEM. To compare observations from different dynamic substrate strains, a two-sample Student's t-test was used in which sample variance was not assumed to be equal. To compare observations from more than two groups, a one-way analysis of variance was employed followed by a Student–Newman–Keuls multiple comparisons post-hoc test (Instat, GraphPad Software, Inc., San Diego, CA). $p<0.05$ was considered statistically significant.

Results

$Ca^{2+}{}_i$ Responses to Different Substrate Strains. Typical cell $Ca^{2+}{}_i$ responses are shown in Fig. 3. The fraction of hFOB

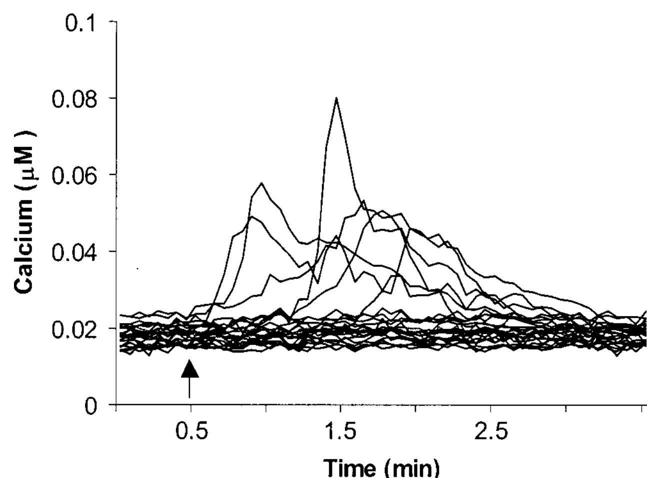

Fig. 3 An example of the hFOB cell $[Ca^{2+}]_i$ response traces obtained for oscillating flow (2 N/m^2, 1 Hz). Note that the arrow depicts the onset of flow.

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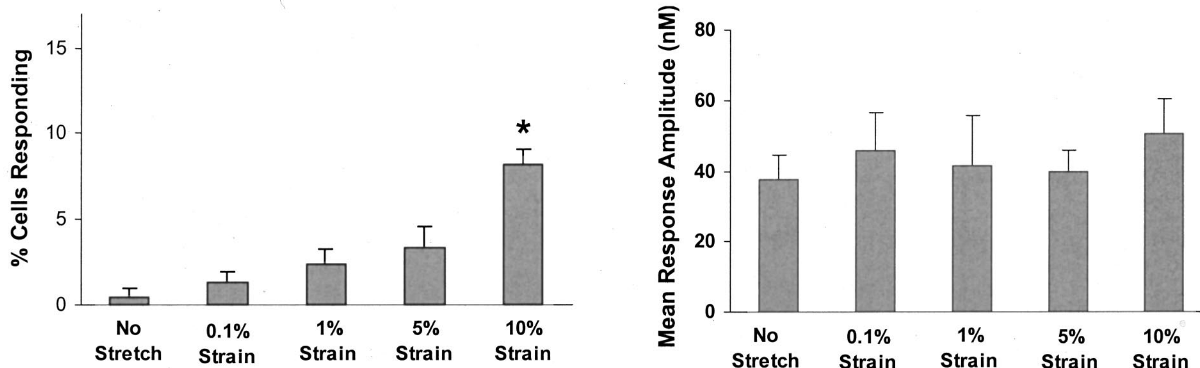

Fig. 4 Fraction of hFOB cells responding with an increase in $[Ca^{2+}]_i$ at different substrate strains and the mean response amplitude. 0.48±0.48 percent, 1.31±0.64 percent, 2.34±0.96 percent, 3.36±1.18 percent and 8.15±0.95 percent of hFOB cells responded for no stretch, 0.1, 1, 5, and 10 percent strain, respectively. Mean response amplitudes of hFOB cells were 37.53±6.83, 45.60±10.81, 41.68±14.00, 39.63±6.32, and 50.57±9.91 nM for no stretch, 0.1, 1, 5, and 10 percent strain, respectively. The data were obtained from six individual experiments and a total of 246 cells. (* represents statistically significant difference ($p<0.05$) from other four groups, no stretch, 0.1, 1, and 5 percent strain).

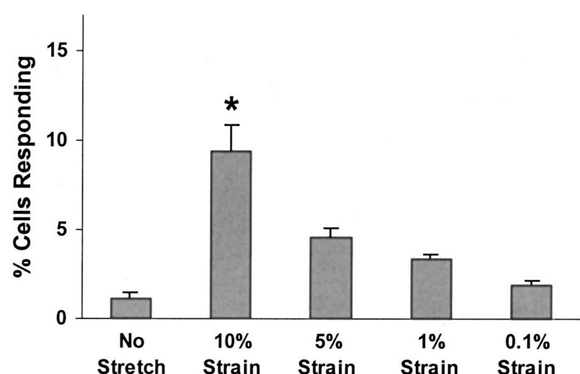

Fig. 5 Fraction of hFOB cells responding with an increase in $[Ca^{2+}]_i$ at the reversing ordering of application of the various substrate strains. 1.16±0.32, 9.40±1.46, 4.57±0.50, 3.40 ±0.22, and 1.90±0.29 percent of hFOB cells responded for no stretch, 10, 5, 1, and 0.1 percent strain, respectively. The data were obtained from four individual experiments and a total of 229 cells. (* represents statistically significant difference ($p<0.05$) from other four groups, no stretch, 5, 1, and 0.1 percent strain).

cells responding with an increase in $Ca^{2+}{}_i$ at different substrate strains is shown in Fig. 4. The data were obtained from six individual experiments (slides) and a total of 246 cells. The percentage of cells responding to substrate strain levels expected to occur during routine physical loading (0.1 percent, 1 Hz) was not statistically different from the percentage of cells exhibiting spontaneous $Ca^{2+}{}_i$ transients during the nonstretch control period (1.31 ±0.64 percent and 0.48±0.48 percent, respectively). When strains were increased to 1 percent and 5 percent, the percentage of cells responding increased to 2.34±0.96 percent and 3.36 ±1.18 percent, respectively. However, these changes were not significantly different from the nonstretch control period. At a strain of 10 percent, the percentage of cells responding increased to 8.15±0.95 percent, which was significantly greater than all the other strain levels ($p<0.05$). Mean response amplitudes are shown in Fig. 4. The amplitudes of 37.53±6.83 nM, 45.60 ±10.81 nM, 41.68±14.00 nM, 39.63±6.32 nM, and 50.57 ±9.91 nM ($p>0.05$) were not statistically different from one another. The fraction of hFOB cells responding with an increase in $Ca^{2+}{}_i$ to the reversed order of strain application is shown in Fig. 5. 10 percent dynamic strain still induced statistically significant $Ca^{2+}{}_i$ responses compared with all other cases. The results for

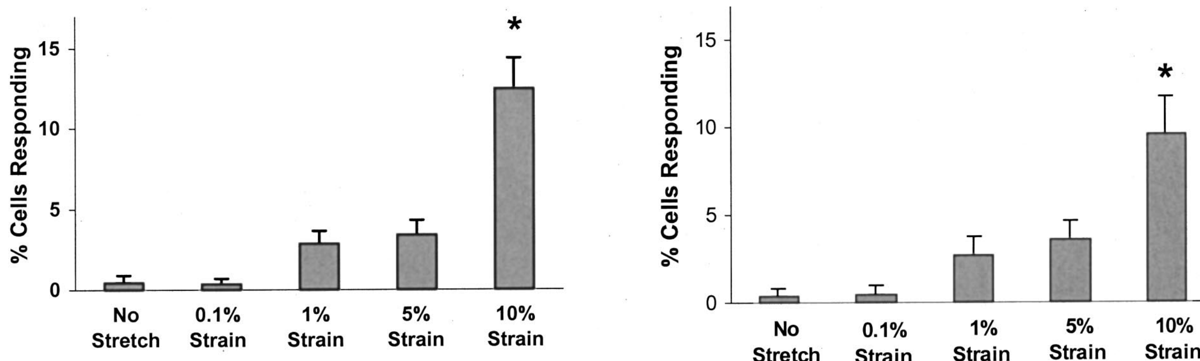

Fig. 6 Fraction of cells responding with an increase in $[Ca^{2+}]_i$ at different substrate strains for ROB cells (left) and MLO-Y4 cells (right). 0.45±0.45, 0.35±0.35, 2.85±0.79, 3.30±0.90, and 12.46±1.91 percent were the percentages of ROB cells responding for no stretch, 0.1, 1, 5, and 10 percent strain. Only the response for 10 percent strain was significantly different from those of four other cases. The results for ROB cells were from six individual experiments that contained total 293 cells. The percentages of responding MLO-Y4 cells were 0.39±0.39, 0.48±0.48, 2.72±1.04, 3.55±1.15, and 9.53±2.17 percent for no stretch, 0.1, 1, 5, and 10 percent strain. The response of 10 percent strain was significantly different from those for 0.1, 1, and 5 percent strain. Six individual experiments had total 227 MLO-Y4 cells. (* represents statistically significant difference ($p<0.05$) from other four groups, no stretch, 0.1, 1, and 5 percent strain).

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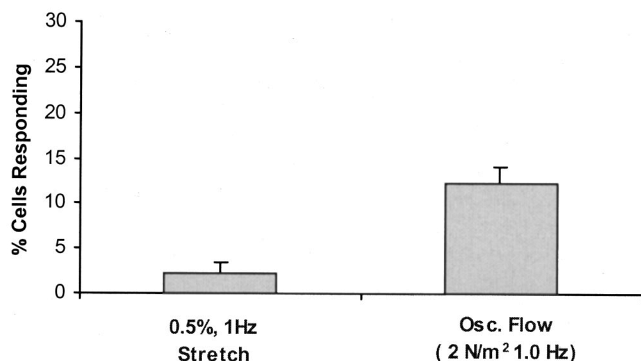

Fig. 7 Fraction of cells responding with an increase in $[Ca^{2+}]_i$ to dynamic substrate strain (0.5 percent, 1 Hz) and oscillatory flow (2 N/m^2, 1 Hz) for hFOB cells. The percentage numbers were 2.24±1.28 percent for dynamic strain and 12.30±1.88 percent for oscillating flow. The total cell numbers for strain and flow were 122 and 332, respectively.

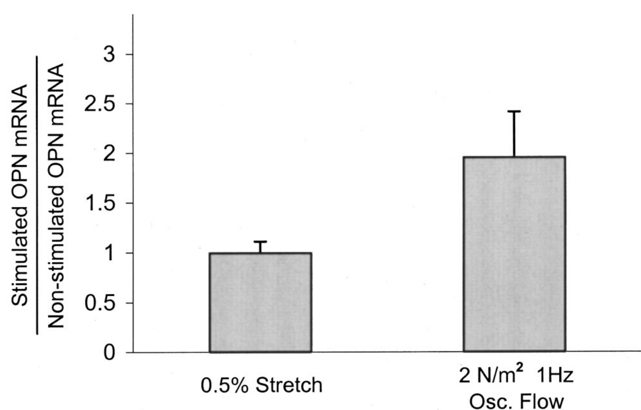

Fig. 8 The relative hFOB cell osteopontin mRNA level change in response to physical stimuli: substrate deformation 0.99 ±0.12 (*n*=3) and oscillating fluid flow 1.95±0.40 (*n*=2). The mRNA level was measured 72 hours after stimulation.

ROB and MLO-Y4 cells exposed to varying levels of substrate strain were similar to that obtained for hFOB cells (Fig. 6). As with the hFOB cells, a statistically significant response was found only for a 10 percent strain. The mean response amplitudes of ROB and MLO-Y4 cells were not significantly different for all cases (data not shown).

$Ca^{2+}{}_i$ Responses to Routine Substrate Strains and Oscillatory Fluid Flow. The levels of substrate strain and oscillatory fluid flow associated with routine physical activity were obtained from the literature [7,13]. The results for hFOB cells are given in Fig. 7. As in the previous experiments, the number of cells that responded to strains of 0.5 percent at 1 Hz was not significantly different from no-stretch control. In contrast, there was a significant ($p<0.05$) increase in the percentage of cells responding to oscillatory flow (2 N/m^2, 1 Hz).

Osteopontin mRNA Level. Osteopontin mRNA levels in hFOB were quantified in response to 0.5 percent substrate strain at 1 Hz and oscillating fluid flow at 1 Hz, resulting in a wall shear stress of 2 N/m^2 utilizing QRT RT-PCR. There was no statistically significant change in osteopontin mRNA level in response to 0.5 percent stretch (Fig. 8) compared to nonstretch controls. The relative change in hFOB osteopontin mRNA level was 0.99 ±0.12 ($n=3$). However, oscillating fluid flow increased the osteopontin mRNA level nearly 100 percent (1.95±0.40, $n=2$) relative to nonstretch controls.

Discussion

Whether mechanical adaptation of bone occurs as a direct response to strain, or indirectly as a response to strain-induced fluid flow remains a controversial subject. A number of studies of the biological effect of strains above 0.5 percent can be found in the literature [4–6,31]. However, there are few studies restricted to the effects of strain levels expected to occur due to routine mechanical loading (strain <0.5 percent [7,10,15]). In this study a novel, precisely controlled membrane stretch device was employed to assess the response of osteoblastic cells to varying mechanical strains (0.1–10 percent, 1 Hz) without inducing fluid flow. The effect of oscillatory fluid flow on bone cells was studied with a parallel plate flow chamber system [17] at levels expected to occur in the lacunar–canalicular system due to routine physical activity. These two methods give us the ability to separate the effects of substrate deformation from those of fluid flow. Focusing on physical signals that may occur due to routine physical activities is critical to fully understanding the mechanism of mechanotransduction in bone cells *in vivo*.

Our data demonstrate that continuum strain levels observed to occur in bone during routine physical activities do not induce $Ca^{2+}{}_i$ responses in human osteoblastic cells (hFOB), primary cultures of rat bone cells (ROB) or osteocytic cells (MLO-Y4) *in vitro*. There was no significant difference ($p>0.05$) in the percentage of cells responding in the no stretch period compared with 0.1 percent substrate strain at 1 Hz for all three cell types. Larger strains (1–5 percent) induced responses only in approximately 5 percent of total cells, which were still not significantly different from control ($p>0.05$). When the strain reached 10 percent, the number of cells exhibiting a $Ca^{2+}{}_i$ response increased significantly. The difference between 10 percent stretch and all other situations (no stretch, 0.1 percent, 1 percent or 5 percent stretch) was significant ($p<0.001$). The results of hFOB cells responding with an increase in $Ca^{2+}{}_i$ to the reversed order of application of strain application indicate that the $Ca^{2+}{}_i$ responses for each strain level were independent and not cumulative. We observed differences in $Ca^{2+}{}_i$ responsiveness, in terms of the fraction of responding cells, which were not paralleled by response amplitude. Thus, there was no relationship between $[Ca^{2+}]_i$ oscillation amplitude and the magnitude of substrate stretch. This suggests that the bone cell $Ca^{2+}{}_i$ response to substrate deformation is an all-or-none response and that bone cell sensitivity to loading intensity is expressed in terms of the number of responding cells rather than their $[Ca^{2+}]_i$ oscillation amplitude. This is consistent with observations of the $Ca^{2+}{}_i$ response of bovine aortic endothelial cells [32], articular chondrocytes [33] and bone cells [17,18] to fluid flow. Our data support the concept that there may be a threshold for bone cell response to substrate strain *in vitro*, and are in agreement with the conclusion of Owan et al. [10], that this threshold appears to be between 5 and 10 percent. Our results are also consistent with previous studies [4–6,31], which employed strain at or above 5 percent. However, *in vivo* data [7] have shown that continuum bone tissue strains remain less than 0.5 percent under routine conditions of physical activity. Indeed, tissue strains in excess of this level are associated with tissue failure. High strain magnitudes (5 percent and higher) can occur in pathologic conditions such as fracture. Thus bone cell mechanotransduction may involve two distinct pathways. The first pathway would play a role in bone tissue adaptation and homeostasis and would be mediated by loading-induced oscillating fluid flow as a cellular physical signal, but not substrate stretch. The second pathway would be activated in fracture healing and repair and involve direct cellular sensitivity to large substrate deformation.

In addition to substrate strain, mechanical loading of bone tis-

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sue has been shown to increase fluid flow through the lacunar–canalicular spaces [34]. Therefore, in addition to examining the dose response of bone cell $[Ca^{2+}]_i$ to substrate strain, we also quantified the $[Ca^{2+}]_i$ and OPN mRNA responses to substrate strain relative to fluid flow regimes expected to occur due to typical physical activities. Lacunar–canalicular flow occurs due to two phenomena, arterial pressure that leads to steady flow, and mechanical loading that leads to oscillating flow. Loading-induced fluid flow is oscillatory due to the cyclic nature of the applied loads. When load is applied to bone, fluid flows from regions of relative high strain to regions of low relative strain, and reverses direction when the load is removed [35]. In this study, oscillating fluid flow resulting in a wall shear stress of 2 N/m^2 applied at 1 Hz induced $Ca^{2+}{}_i$ responses that were similar to those for 10 percent dynamic substrate strain at 1 Hz. Furthermore, dynamic strain at 0.5 percent and 1 Hz did not alter OPN mRNA level, but oscillating flow almost doubled the level of OPN mRNA. Furthermore, these results are consistent with the findings of Harter et al. [22] and Toma et al. [6] who found osteogenic changes in OPN expression only for strains in excess of 1 percent. Although the responsiveness of bone cells to steady flow has been reported to be greater than that to oscillatory flow [17], oscillatory flow is the predominant flow regime *in vivo* [16]. Moreover, relative to routine dynamic strain levels, our data suggest that routine levels of oscillating fluid flow may be more stimulatory to bone cells, possibly because fluid flow may result in more cellular deformation than substrate stretch.

This study has focused on determining the aspects of the physical environment of the cell involved in the response of bone tissue to routine mechanical loading. Two biological outcome variables were quantified, $Ca^{2+}{}_i$ mobilization and osteopontin mRNA level. $Ca^{2+}{}_i$, is a ubiquitous second messenger that controls a number of cellular responses [36–38], and, as such, may have an important role in the mechanotransduction mechanism for bone cells [17,18,39,40]. In addition, $Ca^{2+}{}_i$ mobilization is one of the earliest notable intracellular signals we can explore. Osteopontin (OPN) is one of the major noncollagenous proteins found in bone extracellular matrix and is considered to play an important role in bone formation, resorption and remodeling [21,41–43]. Thus, $[Ca^{2+}]_i$ and OPN expression have both been implicated in the literature to play important roles in the biochemistry of bone cell mechanotransduction. In our study they have been treated as two, possibly independent, biologic endpoints that span a range from relatively early to relatively late changes in bone cell metabolism. We do not, however, suggest that they are necessarily part of a single biochemical pathway.

Indeed, while our results suggest that oscillating fluid flow may be a more significant physical signal relative to substrate deformation in cellular sensing of routine physical activities, the biochemical mechanotransduction mechanism remains unclear. For example, both extracellular and intracellular Ca^{2+} sources are known to contribute to the $Ca^{2+}{}_i$ response to steady fluid flow in bone cells and chondrocytes [18,44]. It is possible that the Ca^{2+} sources contributing to $Ca^{2+}{}_i$ response we have observed for substrate deformation and oscillating fluid flow may be different. However, the significance of such an experiment is limited by our finding of a lack of a $Ca^{2+}{}_i$ response to continuum strain at levels observed to occur during routine physical activity. Identification of the source of $Ca^{2+}{}_i$ in the response to oscillating fluid flow as well as linking the $Ca^{2+}{}_i$ and OPN responses are important aims for future experiments, but not consistent with the focus of our current study, namely extracellular physical signals. Our results suggest that oscillating fluid flow is an appropriate physical signal for future investigations of the biochemical mechanotransduction pathway.

Some limitations should be acknowledged when interpreting our results. First, although accurate data are available concerning the continuum level of strain resulting from routine physical activities, no direct experimental quantification of lacunar–canalicular flow currently exists in the literature. We have based our applied flow regime on the best currently available scientific evidence. Specifically, our flow protocol is based on theoretical predictions that have been validated with respect to experimental measurements of flow-induced streaming potentials [13]. Also, due to the two different experimental apparatuses utilized in this study, the substrates upon which cells were cultured were different between dynamic substrate stretch and fluid flow experiments. In the hFOB and ROB stretch experiments the substrate was fibronectin coated silicone membranes versus uncoated quartz glass for the flow experiments. However, both published [17] and unpublished experiments in our laboratory have not found a significant effect on bone cell $[Ca^{2+}]_i$ sensitivity of a fibronectin coating versus uncoated quartz glass slides. All MLO-Y4 experiments were conducted with type I collagen coated substrates to maintain the osteocytic phenotype. Finally, the waveforms of the two devices were subtly different due to the technical capabilities of the two systems. A triangle wave was utilized in the strain experiments and a sine wave utilized in the flow experiments.

In summary, using our novel substrate stretch device and fluid flow chamber, we have been able to elucidate the effects of substrate deformation and fluid flow on bone cells independently. This study represents the first published results for the dose-response effect of dynamic substrate strains on cytosolic calcium mobilization and oscillatory fluid flow on OPN mRNA levels. Our data suggest that the continuum levels of dynamic substrate strain observed to occur due to routine physical activities do not increase $[Ca^{2+}]_i$ or OPN mRNA levels *in vitro*. However, a fluid flow regime predicted to occur due to routine physical loading increased both $[Ca^{2+}]_i$ and OPN mRNA levels. Therefore, relative to fluid flow, substrate deformation may play less of a role as a physical signal in bone cell mechanotransduction of routine loading.

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