Osteoblasts respond to pulsatile fluid flow with short-term increases in PGE2 but no change in mineralization

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Nauman, E. A., R. L. Satcher, T. M. Keaveny, B. P. Halloran, and D. D. Bikle. Osteoblasts respond to pulsatile fluid flow with short-term increases in PGE2 but no change in mineralization. J Appl Physiol 90: 1849–1854, 2001.—Although there is no consensus as to the precise nature of the mechanostimulatory signals imparted to the bone cells during remodeling, it has been postulated that deformation-induced fluid flow plays a role in the mechanotransduction pathway. In vitro, osteoblasts respond to fluid shear stress with an increase in PGE2 production; however, the long-term effects of fluid shear stress on cell proliferation and differentiation have not been examined. The goal of this study was to apply continuous pulsatile fluid shear stresses to osteoblasts and determine whether the initial production of PGE2 is associated with long-term biochemical changes. The acute response of bone cells to a pulsatile fluid shear stress (0.6 ± 0.5 Pa, 3.0 Hz) was characterized by a transient fourfold increase in PGE2 production. After 7 days of static culture (0.00.5 Pa, 3.0 Hz) was characterized by a transient fourfold response of bone cells to a pulsatile fluid shear stress (0.6 ± 0.5 Pa, 3.0 Hz) was characterized by a transient fourfold increase in PGE2 production; however, the long-term effects of fluid shear stress on cell proliferation and differentiation have not been examined. The goal of this study was to apply continuous pulsatile fluid shear stresses to osteoblasts and determine whether the initial production of PGE2 is associated with long-term biochemical changes. The acute response of bone cells to a pulsatile fluid shear stress (0.6 ± 0.5 Pa, 3.0 Hz) was characterized by a transient fourfold increase in PGE2 production. After 7 days of static culture (0.00.5 Pa, 0.3 Hz) or low (0.06 ± 0.05 Pa, 0.3 Hz) or high (0.6 ± 0.5 Pa, 3.0 Hz) levels of pulsatile fluid shear stress, the bone cells responded with an 83% average increase in cell number, but no statistical difference (P > 0.53) between the groups was observed. Alkaline phosphatase activity per cell decreased in the static cultures but not in the low- or high-flow groups. Mineralization was also unaffected by the different levels of applied shear stress. Our results indicate that short-term changes in PGE2 levels caused by pulsatile fluid flow are not associated with long-term changes in proliferation or mineralization of bone cells.

bone remodeling; parallel-plate flow chamber; tissue engineering

IT IS WELL ESTABLISHED THAT mechanical loading is capable of producing a complex adaptive response in bone tissue (7, 11, 14, 22, 35). Forces exerted at the whole bone level are transduced into inhomogeneous strain fields, pulsatile fluid flows, and both chemical and electrical potential gradients at the cellular level (4, 16, 41, 42, 44). In vivo experiments suggest that strain magnitude, strain rate, strain gradients, strain energy density, anatomic site, and biochemical environment are all important factors in the response of bone to mechanical loading (6, 12, 27, 29, 35, 42, 43). It remains unclear how these various signals influence long-term integrated biochemical responses such as mineralization and bone remodeling, but it has been hypothesized that fluid shear stresses play a prominent role in the mechanotransduction pathway (16, 41, 44).

Parallel-plate flow chambers have been used to examine the effects of flow-induced shear stresses on the biochemical response of osteoblasts primarily because they provide a means of delivering a well-controlled mechanical stimulus to a relatively homogenous population of cells (9, 20, 28, 36, 40). The short- and intermediate-term (<8 h) response of osteoblasts to steady fluid shear stresses involves an increase in the intracellular calcium concentration (17–19) and the production of prostaglandin E2 (PGE2) (24, 32, 33). In vivo studies have shown that PGE2 administration has an anabolic effect on bone formation (21, 30, 39). Consequently, it is often assumed that increases in PGE2 production presage increased cell proliferation and mineralization, but, to date, this has not been studied in vitro. Unidirectional pulsatile flow has also been shown to decrease alkaline phosphatase (AP) expression relative to steady flow or static culture (15). Because AP expression is thought to decrease just before the onset of mineralization, these results suggest that pulsatile fluid flow may stimulate long-term bone formation. In support of this theory, cells grown for 25 days on porous collagen beads and subjected to shear stresses on the order of 1 dyn/cm2 increased matrix and mineral accumulation compared with static cultures (16). The increase in mineralization at day 25 was presaged by a decrease in AP activity at days 11 and 18 (16), but to date there has been no comparison between the short-term (<24 h) anabolic response and the long-term (≥7 days) effects of fluid flow on mineralization.

The overall goal of this study was to apply well-defined pulsatile fluid shear stresses of various magnitudes to monolayer cultures of bone cells in vitro and
measure both the short-term anabolic response and long-term mineralization. Specifically, we performed two experiments. In the first, we exposed bone marrow stromal cell-derived osteoblast cultures to average shear stresses of 0 and 0.6 Pa and measured PGE2 production and AP activity within the first 48 h. In the second experiment, we subjected osteoblasts to average shear stresses of 0, 0.06, and 0.6 Pa and measured the long-term (7 days) effects on cell proliferation, AP activity, and mineralization.

METHODS

Rat tibial and femoral osteoprogenitor cells were harvested by using techniques described in detail elsewhere (5, 25, 37, 38). Briefly, two 6-wk-old male Sprague-Dawley rats were killed, and both tibiae and femora were excised. After removal of the soft tissue, the bones were rinsed several times in a solution of phosphate buffered saline (PBS), antibiotics (penicillin-streptomycin), and fungizone under sterile conditions. The epiphyses of each bone were removed, and the diaphysis was suspended in a porus-bottomed oval receptacle within a microcentrifuge tube (5). The tube was filled with primary culture medium [aMEM with nucleosides, 10% fetal bovine serum, 1% antibiotics (penicillin and streptomycin), 0.1% fungizone, 50 μg/ml ascorbic acid, and 5 ng/ml basic fibroblast growth factor (bFGF)] and centrifuged for 5 min at ~8,000 g. The marrow was collected from the bottom of each tube and mixed together with an additional volume of primary culture medium. The solution was repeatedly drawn through a pipette and then an 18-gauge needle to mechanically dissociate the mixture into a uniform single cell suspension. The cells were plated at ~400,000 cells/cm² on 6.4 x 6.4 cm square tissue culture plates coated with cross-linked gelatin (10). On day 5, the primary medium was replenished, and, subsequently, the cells were fed three times per week with secondary medium (aMEM with nucleosides, 10% fetal bovine serum, 1% antibiotics, 0.1% fungizone, 50 μg/ml L-ascorbic acid, and 3 mM β-glycerophosphate). Colonies began to mineralize after 12–14 days in culture. On day 14, four plates were loaded into parallel-plate flow chambers (Fig. 1) (9, 28) and connected to separate reservoirs (Fig. 2) as described in detail elsewhere (23, 28).

The first experiment was designed to examine the short-term response of bone cells to mechanical loading. The cells were subjected to an average shear stress of 0.6 Pa (pulsed between 0.1 and 1.1 Pa at a frequency of 3.0 Hz) because this level has been shown to substantially elevate PGE2 levels for up to 8 h in neonatal rat calvariae-derived osteoblasts (32, 33). Although many in vivo studies apply a fixed number of loading cycles per day (8, 42), for the purposes of this experiment, flow-induced shear stresses were applied continuously for up to 48 h. This was done, in part, to simplify the already challenging experimental protocol, as well as to remove waste products and maintain sufficient oxygenation. Supernatant samples were removed from each reservoir of the stressed cells and the control wells at 0, 2, 4, 6, 24, and 48 h for PGE2 quantification using an ELISA assay (Amersham Pharmacia Biotech, Piscataway, NJ). One plate from each group was taken at 2, 6, 24, and 48 h, fixed in 10% neutral buffered formalin, and assayed for AP activity and cell num-

Fig. 1. Schematic of a parallel-plate flow chamber. Cells were grown on a polystyrene plate (A) for 14 days and then sealed to the opposing face of the flow chamber with a silicone gasket placed in the gasket channel (B). Cell culture medium enters through the inlet port (C), flows through the inlet tube (D), and enters the flow field through the inlet slit (E). The fluid exits the flow field through the outlet slit (F) and collects in the outlet tube (G). After it moves through the outlet port (H), the fluid returns to the medium reservoir.

Fig. 2. Schematic of the flow loop. A peristaltic pump moves fluid from the lower reservoir directly to the inlet of the flow chamber and then back to the upper reservoir. The pump controls the average flow rate, and the cyclic compression of the tubing by the pump's rollers transmits a pulsed flow to the cells in the flow chamber. Arrows indicate the direction of the bulk fluid movement.
The second experiment was designed to determine whether bone cells respond to elevated levels of fluid flow in a dose-dependent manner. There were four experimental groups: the time 0 static controls (n = 10), low-flow (mean shear stress = 0.06, pulsed between 0.01 and 0.11 Pa, at a frequency of 0.3 Hz) group (n = 6), high-flow (mean shear stress = 0.6, pulsed between 0.1 and 1.1 Pa at a frequency of 3.0 Hz) group (n = 7), and day 7 static controls (n = 10). In each of the flow groups, pulsatile fluid shear stresses were applied continuously. After 7 days of flow, the cells were assayed for AP activity, cell number, and mineralization.

PGE₂ production. PGE₂ production was determined via a competitive enzyme immunoassay kit (Amersham Pharmacia Biotech). Briefly, 50 μl of the cell medium aspirate were mixed with PGE₂ peroxidase conjugate and a PGE₂ antibody and incubated at room temperature for 1 h in a specially coated microtiter plate. The PGE₂ in the sample and the PGE₂ peroxidase conjugate both compete for a finite number of binding sites on the PGE₂ antibody. After the wells were aspirated and rinsed, an enzyme substrate was used to develop the color in the bound PGE₂ peroxidase conjugate. Subsequently, 1 M sulfuric acid was added to the wells, and the resulting yellow color was read in a spectrophotometer at 450 nm.

AP activity. AP activity was determined using commercially available standard reagents. Cells were incubated for 10 min at 37°C in a solution consisting of equal parts p-nitrophenol phosphate (Sigma 104; Sigma Chemical, St. Louis, MO) and alkaline buffer solution 221 (Sigma Chemical). The absorbance of the resulting solution was measured with a spectrophotometer at 410 nm (A 410).

Cell number determination. Cell cultures were stained with a solution of 0.2% crystal violet in 2% ethanol for 30 min at room temperature. The solution was aspirated, and the

![Fig. 3](http://jap.physiology.org/) - Combined results of 3 separate experiments illustrate the transient increase in PGE₂ production per cell resulting from a pulsatile fluid shear stress of 0.6 Pa. Values are means ± SE. PGE₂ levels in the flow group began to increase at 2 h, peaked at 6 h, and returned to their initial levels after 48 h. A 590, absorbance measured at 590 nm.

![Fig. 4](http://jap.physiology.org/) - Combined results of 3 separate experiments illustrate the short-term alkaline phosphatase (AP) activity. Values are means ± SE. AP activity per cell did not depend on the applied fluid shear stress (P > 0.29) but did decrease significantly with time (P < 0.02). There was no interaction effect (P > 0.20).

![Fig. 5](http://jap.physiology.org/) - Effects of pulsatile fluid flow on cell number normalized to the day 14 values. All cells were grown in culture without flow for 14 days and subsequently exposed to an average shear stress of 0.0, 0.06, or 0.6 Pa. After 7 days of exposure, there was a 67, 102, and 91% increase, respectively, in cell number (*P < 0.035 vs. day 14 controls). The magnitude of the applied shear stress did not affect cell number (P > 0.53).
wells were rinsed four times with distilled water to remove unbound stain. The crystal violet solution was eluted with 0.2% Triton X-100 (Sigma Chemical), and the absorbance was measured with a spectrophotometer at 590 nm.

Mineralization. Cell cultures were stained with 2% alizarin red for 10 min and rinsed three times with distilled water. The remaining stain was bound only to the calcium salts in the matrix. The stain was eluted with 10% cetylpyridinium chloride for 2 h, and the total absorbance was measured with a spectrophotometer at 540 nm. Alizarin red staining is a sensitive technique for measuring the degree of mineralization in osteoblast cultures and provides a linear relationship between the measured absorbance and total mineralization (25, 26).

Statistical analysis. Data are presented as means ± SE. The PGE2 and AP levels in experiment 1 were normalized by the cell number of the corresponding plate. In the second experiment, all the output variables in each group were normalized to the average for the day 14 static controls. Outliers were detected and removed according to the criteria set forth by Grubbs (13), and the data were analyzed by analysis of variance (ANOVA) and a post hoc Tukey’s test (Systat version 5.2, Evanston, IL).

RESULTS

Short-term (48 h) experiments. In response to an average fluid shear stress of 0.6 Pa, bone cells exhibited a transient fourfold increase in PGE2 production per cell that peaked at 6 h and returned to basal levels by 48 h (Fig. 3). A two-factor ANOVA indicated that the presence or absence of fluid flow was the only significant variable ($P < 0.0001$). In contrast, AP activity per cell (Fig. 4), a measure of osteoblast differentiation, exhibited a significant decrease with time ($P < 0.02$) but was not dependent on the presence of fluid shear stress ($P > 0.29$).

Long-term (7 day) experiments. After 7 days of exposure to an average shear stress of 0, 0.06, or 0.6 Pa, the bone cells responded with a 67, 102, and 91% increase, respectively, in cell number with respect to their pre-flow levels (Fig. 5). Although cell number increased in each of the day 21 groups compared with the day 14 controls ($P < 0.035$), flow had no statistically significant effect on cell number ($P > 0.53$). Total AP activity per cell decreased at 21 days ($P = 0.044$) in the static cultures but was sustained in cells exposed to flow (Fig. 6). Total mineralization increased with time, but the changes were not significant ($P > 0.28$) (Fig. 7). The level of flow did not significantly impact the level of mineralization achieved. The power of this statistical test was 0.95, indicating that there was a 5% chance of making a type II error (45).

DISCUSSION

Well-controlled pulsatile fluid shear stresses were applied to in vitro cultures of osteoblasts derived from rat bone marrow stromal cells. The acute increase in PGE2 production first observed by Reich and Frangos (32, 33) in neonatal rat calvarial cells was verified in this cell culture model. This effect did not, however, presage an increase in cell proliferation or mineralization. Cell number increased with time but was unaffected by the application of fluid shear stress. Differentiation, as measured by AP activity, was not affected by the magnitude of the flow-induced shear stresses in Fig. 6. AP activity normalized to day 14 results. All cells were grown in culture without flow for 14 days and subsequently exposed to an average shear stress of 0.0, 0.06, or 0.6 Pa. There was a significant reduction in AP activity per cell in the static group relative to the day 14 controls ($^*P = 0.044$), but the application of fluid shear stress had no statistically significant effect ($P > 0.24$).

Fig. 7. Mineralization normalized to the day 14 results. All cells were grown in culture without flow for 14 days and subsequently exposed to an average shear stress of 0.0, 0.06, or 0.6 Pa. Mineralization increased threefold between days 14 and 21, but, because of the high degree of scatter, there was no significant dependence on time or applied shear stress ($P > 0.28$).
the short-term or long-term experiments. Likewise, there was no detectable effect of flow on total mineralization. Thus the short-term biochemical response of bone cells as measured by PGE$_2$ production is not necessarily a good indicator of their long-term anabolic response in this particular cell culture model.

Strengths of this study include the use of a well-characterized parallel-plate flow chamber (28) to provide controlled pulsatile fluid shear stresses to monolayer cultures of bone cells in vitro and its use to generate long-term (7 days) results. For the short-term experiments, a nominal level of 0.6 Pa was used because it had been shown to induce a substantial PGE$_2$ response in neonatal rat calvarial cells (32, 33). For the long-term experiments, two nominal flow rates were used (0.06 and 0.6 Pa) in an effort to determine the sensitivity of the anabolic response to the level of applied shear stress.

Weaknesses of this study include the relatively small sample size and the need to increase the frequency of the applied fluid shear stresses to increase its magnitude. The time lag between the assembly of the parallel-plate flow chamber, which took place in a sterile laminar flow biosafety cabinet, and the onset of fluid flow in a humidified incubator prevented the use of more than four flow chambers in any given experiment. Consequently, it was necessary to compile the results of five different experiments. Although this resulted in a relatively small sample size ($n = 6–10$) for each experimental group, similar trends were observed between experiments, thereby providing good repeatability and statistical power. In addition, the direct connection between the peristaltic pump and the parallel-plate flow chamber required the frequency of the loading regimen to be directly related to the average applied shear stress. Consequently, the low shear stress group (mean $= 0.06$ Pa) was loaded at 0.3 Hz and the high shear stress group (mean $= 0.6$ Pa) was loaded at 3.0 Hz.

Obtaining data points beyond 7 days may not be possible with this experimental protocol, for a variety of reasons. First, as the bone cells multiply and mineralize, they begin to form three-dimensional colonies whose thickness may become significant relative to the 220-μm gap between the parallel plates, and under these conditions the assumptions of Poiseuille flow are no longer valid. As a result, it would be difficult to predict the shear stresses exerted on the bone cells without resorting to complex computational fluid dynamics analysis. Although these experimental difficulties make direct comparisons to the 25-day study carried out by Hillsley and Frangos (16) using a bioreactor type model difficult, our findings are novel in that they provide long-term data that were previously lacking in earlier bone cell culture models.

The cell culture model used here employed bFGF and β-glycerophosphate, however, it was found that the cells were swept off the substrate over the course of 3–5 days even at low flow rates. Although the concentration of β-glycerophosphate used here (3 mM) is much less than that (10 mM) used in most osteoblast cell culture experiments (1, 31), the interaction effects between these chemical stimuli and flow-induced shear stresses are not known. It is also possible that multiple bouts of loading or a different loading waveform may provide a greater stimulatory effect than one long continuous load such as the one used in this study. These potential variables deserve further investigation.

Osteoblast production of PGE$_2$ in response to fluid shear stress has been observed previously (23, 24, 34, 40). Compared with static controls, osteoblasts and osteocytes obtained from a variety of sources (i.e., neonatal rat calvaria, chick calvaria, and osteosarcoma cell lines) transiently increased their production of PGE$_2$ by 2- to 20-fold when challenged with shear stresses ranging in average magnitude from 0.5–2.4 Pa (23, 24, 34, 40) and this response is mediated by the early production of nitric oxide (23). In the present study we found that bone marrow stromal cell-derived osteoblasts responded to continuous pulsatile fluid shear stresses (mean $= 0.6$ Pa, range $= 0.1–1.1$ Pa) with a maximum fourfold increase in PGE$_2$ production. Although further experiments are needed to better localize the peak PGE$_2$ response, it did not presage an increase in mineralization. These results indicate that additional short-term and long-term experiments are needed to put these results in better perspective and elucidate the biochemical processes responsible for the adaptation of bone to mechanical loading.

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