Influence of increased mechanical loading by hypergravity on the microtubule cytoskeleton and prostaglandin E$_2$ release in primary osteoblasts

Nancy D. Searby,1,2 Charles R. Steele,2 and Ruth K. Globus1,3
1Life Sciences Division, National Aeronautics and Space Administration Ames Research Center, Moffett Field; 2Department of Mechanical Engineering, Stanford University, Stanford; and 3Department of Stomatology, University of California, San Francisco, San Francisco, California

Submitted 22 November 2003; accepted in final form 16 February 2005

Searby, Nancy D., Charles R. Steele, and Ruth K. Globus. Influence of increased mechanical loading by hypergravity on the microtubule cytoskeleton and prostaglandin E$_2$ release in primary osteoblasts. Am J Physiol Cell Physiol 289: C148–C158, 2005. First published February 23, 2005; doi:10.1152/ajpcell.00524.2003.—Cells respond to a wide range of mechanical stimuli such as fluid shear and strain, although the contribution of gravity to cell structure and function is not understood. We hypothesized that bone-forming osteoblasts are sensitive to increased mechanical loading by hypergravity. A centrifuge suitable for cell culture was developed and validated, and then primary cultures of fetal rat calvarial osteoblasts at various stages of differentiation were mechanically loaded using hypergravity. We measured microtubule network morphology as well as release of the paracrine factor prostaglandin E$_2$ (PGE$_2$). In immature osteoblasts, a stimulus of 10$^{-5}$ gravity (10 g) for 3 h increased PGE$_2$ 2.5-fold and decreased microtubule network height 1.12-fold without affecting cell viability. Hypergravity (3 h) caused dose-dependent (5–50 g) increases in PGE$_2$ (5.3-fold at 50 g) and decreases (1.26-fold at 50 g) in microtubule network height. PGE$_2$ release depended on duration but not orientation of the hypergravity load. As osteoblasts differentiated, sensitivity to hypergravity declined. We conclude that primary osteoblasts demonstrate dose- and duration-dependent sensitivity to gravitational loading, which appears to be blunted in mature osteoblasts.

LIFE AS WE KNOW IT HAS EVOLVED under the influence of Earth’s gravity. Changes in other physical conditions in the environment (e.g., temperature) have profound effects on cells, and gravity may act at a fundamental level as well. Gravity is ubiquitous and influences the mechanical environment within tissues by affecting cell weight, extracellular hydrostatic pressure, and fluid convection. Despite the central importance of gravity, little is known about how it influences cellular physiology.

Because the weight of a cell depends on the amount of gravity acting on its mass, changes in gravity may serve as mechanical stimuli to adherent cells. By applying increased gravity to cells (hypergravity), the direction and magnitude of gravity can be varied to provide insight into its effects on cellular physiology. Coordinated shape and cytoskeletal changes may result from shifts in the position of organelles of different densities within the cell. In a gravity field, the denser nucleus (26, 48, 49) may shift downward, pulling the interconnected cytoskeleton and plasma membrane with it, resulting in a reduced cell height that would be expected to continue to decrease as the gravity field increased. Microtubules are thought to resist compressive loads (21, 22) and may themselves be compressed as a result of the hydrostatic pressure generated by extracellular fluid. Studies of osteoblasts and endothelial cells have shown that reorganization of the cytoskeleton correlates with increased release of prostaglandin E$_2$ (PGE$_2$), which is important for paracrine and autocrine signaling (42, 50). Whether gravity loading results in microtubule rearrangements related to PGE$_2$ release is unclear.

Bone-forming osteoblasts play a mechanosensing role in vivo (9); thus we anticipated that they would be responsive to changes in the gravity vector. In vivo PGE$_2$ stimulates bone formation and resorption and may mediate the response to mechanical loading (38). Osteoblasts respond to substrate deformation, fluid-induced shear stress, and hydrostatic pressure with changes in cell shape, cytoskeletal organization, and PGE$_2$ production (1–3, 7, 10, 12, 19, 27, 32, 34, 37), but less is known about the influence of gravity. When gravity is decreased, such as in the microgravity environment of spaceflight, MC3T3-E1 osteoblasts adopt a more rounded morphology; yet, when corrected for cell number, PGE$_2$ release is unchanged (20). Rat osteosarcoma (ROS 17/2.8) osteoblastic cells subjected to alternating gravity loading between microgravity and 2 g via parabolic aircraft flights exhibit increased cell shape irregularity, decreased cell area, and increased PGE$_2$ (17). Neither of these two previous studies addressed possible changes in three-dimensional shape. Hypergravity increases PGE$_2$ production from MC3T3-E1 osteoblast-like cells, but the relationship to cell shape or cytoskeletal changes has not been studied (14, 28, 31). In spaceflight, microtubule polymerization is impaired in intact leukocytes (36) and microtubules do not self-organize in vitro assays (35), suggesting that altered microtubule polymerization or organization may contribute to observed changes in cell shape. Primary osteoblast cultures at progressive stages of differentiation undergo well-defined changes in cell shape (16, 33), and the influence of gravity on cytoskeleton and PGE$_2$ production may differ as a consequence.

Primary osteoblasts offer the advantage that their function and regulation more closely mimic osteoblasts in vivo, which is not always the case with cell lines. Treatment of confluent primary osteoblasts with ascorbic acid (AA) and β-glycerophosphate (β-GP) leads to a progression of events, including proliferation and multilayering, synthesis of an extracellular matrix, and mineralization of that matrix with associated changes in cell shape (5, 33). Spaceflight impairs differentiation of primary embryonic chick osteoblasts (25), suggesting

Address for reprint requests and other correspondence: N. D. Searby, National Aeronautics and Space Administration Ames Research Center, MS/236-7, Moffett Field, CA 94035 (e-mail: nancy.d.searby@nasa.gov).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
that differentiation is sensitive to microgravity. This result and the cell shape changes associated with differentiation suggest that gravity loading may act differently depending on the stage of cell differentiation.

In this study, we tested the hypothesis that hypergravity loading of primary osteoblasts reduces the microtubule network and nuclear height in a dose-dependent manner, and this change in cell shape accompanies increased PGE2 release. Furthermore, we hypothesized that the stage of differentiation of the osteoblast culture influences these responses. To test these hypotheses, we developed and characterized a cell culture centrifuge that reproduces a standard tissue culture environment and therefore is suitable for both short- and long-term experiments. We showed that immature, confluent primary osteoblasts responded to hypergravity with increased PGE2 release, decreased microtubule network height but no measurable change in nuclear height, and no major morphological changes. Observed changes depended on the dose and duration of the hypergravity stimulus and were associated with progressive differentiation.

MATERIALS AND METHODS

Cell culture. Primary osteoblast cells were obtained by performing sequential collagenase digestion with embryonic day 21 fetal rat calvaria as previously described (5), with minor revisions. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA) Ames Research Center. Briefly, cells were subcultured the day after isolation and plated at a density of 36,000 cells/cm² onto 0.2% gelatin-cross-linked (gelatin type A from porcine skin; Sigma, St. Louis, MO) eight-well chamber slides (0.81 cm²/well, Permanox; Naige Nunc International, Naperville, IL) and grown in α-minimal essential medium containing 10% fetal calf serum (medium and serum; Gibco-BRL/Life Technologies, Grand Island, NY) and antibiotics (penicillin-streptomycin and Fungizone; Gibco-BRL) in 5% CO2 at 37°C. Cells were induced to differentiate and further characterized by Komarova et al. (24). Briefly, when cells

Measurement of individual cell density. Experiments to measure the density of an osteoblast cell (to calculate gravity-induced shear stress in cells placed parallel to the gravity vector) were performed with ROS 17/2.8 cells to represent a homogeneous pool of differentiated osteoblasts. ROS cells were cultured in Ham’s F-12 medium (GIBCO-BRL) containing 10% fetal calf serum, l-glutamine (GIBCO-BRL), HEPES, and antibiotics in tissue culture dishes and were passaged once they were 80% confluent. Cells were trypsinized (0.25% trypsin; Gibco-BRL), pelleted by centrifugation, and resuspended in culture medium to determine cell density. The cell density assay was based on the assumption that particles falling through a column of liquid reach a terminal velocity that can be calculated using Stokes flow theory. Terminal velocity is calculated as 
v_\text{t} = \left(1 - \rho_\text{p}/\rho_\text{f}\right)gd_\text{p}^2/(18\nu\rho_\text{f})\text{, where } \rho_\text{p} \text{ is the particle density, } d_\text{p} \text{ is the particle diameter, } g \text{ is gravity, and } \nu \text{ is the kinematic viscosity.}\n
For all experiments described above, a settling study was performed and compared to that of primary osteoblasts (50 µg/ml; Gibco-BRL) to induce matrix formation and with β-GP (3 mM; Sigma) to induce mineralization. Media were changed every 2–3 days. The medium pH was controlled in one of two ways. In the gravity orientation experiments, media were supplemented with HEPES buffer (10 mM, pH 7.4; Gibco-BRL) before centrifugation and the wells were filled to the top with 700 µl of medium and sealed with liquid-tight gaskets. In other experiments, the wells were filled with 650 µl of medium (10 mm medium height) and the lids were vented for gas exchange. No differences were noted to be a result of the method of pH control in these short-term (<6 h) experiments (data not shown). To determine whether this volume or height of medium (larger than normal medium-to-cell ratio) affected the results, experiments were performed with 325 µl of medium (5 mm medium height), with no differences observed (data not shown).

Cell number. Primary osteoblasts were counted in the differentiating cultures using the methods described by Komarova et al. (24). To release the osteoblasts from their matrix at the termination of the experiment, cultures at each time point were treated sequentially with phosphate-buffered saline (PBS) containing 10 mM EGTA and 20 mM HEPES, pH 7.4, for 20 min and then for 60 min at 37°C in 572 U/ml collagenase in 115 mM NaCl, 5.3 mM KCl, 3 mM K2HPO4, 1 mM CaCl2, 30 mM mannitol, 10 mM glucose, 2 g/BSA, and 24 mM HEPES, pH 7.4. An equal volume of 0.25% trypsin in EDTA (1 mM) in Hanks’ balanced salt solution without Ca²⁺ and Mg²⁺ (GIBCO-BRL) was added to the collagenase, and cells were incubated for another 30 min. Dispersed cells were counted using a hemocytometer.

Cell hypergravity stimulation. Hypergravity was applied using the 1-foot diameter centrifuge (1-FDC) at the NASA Ames Center for Gravitational Biology Research (see http://lifesci.arc.nasa.gov/CGBR/1_ft.html). To minimize evaporation of cell culture medium, the eight-well chamber slides were placed in a 10-cm culture dish with an open 35-mm dish containing 2 ml of water. Alternatively, cell orientation experiments used sealed eight-well chamber slides oriented either flat on the platform or normal to the platform and secured to flasks using adhesive tape. The cells were then placed at the center of the swinging bucket to reduce inertial shear and on a rubber pad to reduce vibration.

Immature confluent osteoblasts were exposed to 238 rpm (10 g) for 3 h to establish a baseline response to hypergravity. Dose response was determined at rotation rates ranging from 112 rpm (2.5 g) to 518 rpm (50 g). The applied gravity level was calculated as the resultant of Earth’s 1 g and the centrifugal acceleration. The duration of a 10-g hypergravity stimulus was then varied from 10 min to 6 h, with acceleration to 10 g in 2 min and return to 1 g as a control for the stimulus of acceleration up to constant speed. For each time point <3 h, medium was replaced so that all cells conditioned the medium for 3 h to ensure comparable PGE2 values, e.g., medium was replaced 2 h, 50 min before the 10-min spin. To assess the influence of orientation,
cells were placed on the centrifuge with the gravity vector both perpendicular and parallel to the cell growth substrate. Cells at various times in culture (associated with different stages of differentiation; Refs. 5, 24, and 29) were stimulated with 3-h exposure to 10 or 50 g. Immediately after loading, medium was collected for PGE2 analysis and cells were fixed for immunocytochemistry.

1-Foot-diameter centrifuge: characterization of apparatus. The 1-FDC consists of a tabletop centrifuge (model 6S-6RH; Beckman Coulter), modified to provide lower rotation rates (45–1,000 rpm yielding 1.4–180 g) and environmental monitoring for cultured cells and small organisms. The centrifuge was integrated with a tissue culture incubator (model 3851; Forma-Scientific) to control temperature, humidity, and CO2. Swinging platforms sized for standard multiwell plate (8.9 cm in the radial direction and 13.3 cm in the circumferential direction) maintained the resultant gravity vector perpendicular to the cell layer. Fans circulated the air between the incubator and centrifuge through insulated ducts, and water traps collected condensation. An identical incubator adjacent to the 1-FDC was used for stationary 1-g controls. Environmental data from the integrated centrifuge-incubator system and the 1-g control incubator were displayed on analog data displays and recorded using a data acquisition system.

The 1-FDC was tested to ensure that the environmental conditions within the centrifuge (other than hypergravity) were similar to those within the adjacent control incubator. Temperature and CO2 were measured inside the centrifuge volume adjacent to the centrifuge lid and inside the control incubator at the back of the unit. The temperature for the 1-FDC supply incubator was set 1°C higher than the control incubator to achieve 37°C within the centrifuge chamber.

To characterize the mechanical loading environment within the centrifuge, vibrations were measured on the platform during rotation. A single-axis, high-sensitivity Bruel & Kjaer type 8318 accelerometer (Naerum, Denmark) was placed on one platform, and an equivalent weight was placed on the opposite platform. Data were transmitted from the rotating platform to the stationary data acquisition system via slip rings temporarily mounted on the centrifuge. Acceleration was measured in the direction of the resultant gravity vector when the centrifuge was spinning at 238 rpm (10 g). Data were collected from the control incubator for comparison. Data were collected at a sampling rate of 256 samples/s for 80 s, processed, and expressed as acceleration in units of gravity, root mean squared (rms).

PGE2 production. The amount of PGE2 released by the cells into the medium was measured using a commercial enzyme immunoassay kit (Amersham Pharmacia, Little Chalfont, UK) according to the manufacturer’s protocol. Samples were read at 450-nm wavelength using a SpectraMax 250 microplate reader. Osteocalcin levels in the medium were measured after 24-h serum starvation using a commercial enzyme immunoassay kit (Biomedica, Stoughton, MA) according to the manufacturer’s protocol. Samples were read at 450-nm wavelength using a SpectraMax 250 microplate reader.

Alkaline phosphatase activity, cells were extracted in 1% Triton X-100 in HEPES buffer, pH 7.4, and then sonicated and centrifuged at 14,000 g for 4 min. Supernatants were stored at −80°C until analysis. A reaction buffer, pH 7.4, composed of 100 mM glycine buffer, 1 mM MgCl2, and 1 mM ZnCl2 in distilled water was added with 60 mM p-nitrophenyl phosphate (Sigma) to the cell supernatants, and then alkaline phosphatase was measured spectrophotometrically (SpectraMax 250 microplate reader) at 405-nm wavelength. Osteocalcin levels in the medium were measured after 24-h serum starvation using a commercial enzyme immunoassay kit (Biomedica, Stoughton, MA) according to the manufacturer’s protocol. Samples were read at 450-nm wavelength using a SpectraMax 250 microplate reader.

To characterize the mechanical loading environment within the centrifuge, vibrations were measured on the platform during rotation. A single-axis, high-sensitivity Bruel & Kjaer type 8318 accelerometer (Naerum, Denmark) was placed on one platform, and an equivalent weight was placed on the opposite platform. Data were transmitted from the rotating platform to the stationary data acquisition system via slip rings temporarily mounted on the centrifuge. Acceleration was measured in the direction of the resultant gravity vector when the centrifuge was spinning at 238 rpm (10 g). Data were collected from the control incubator for comparison. Data were collected at a sampling rate of 256 samples/s for 80 s, processed, and expressed as acceleration in units of gravity, root mean squared (rms).

PGE2 production. The amount of PGE2 released by the cells into the medium was measured using a commercial enzyme immunoassay kit (Amersham Pharmacia, Little Chalfont, UK) according to the manufacturer’s protocol. Samples were read at 450-nm wavelength using a SpectraMax 250 microplate reader. Osteocalcin levels in the medium were measured after 24-h serum starvation using a commercial enzyme immunoassay kit (Biomedica, Stoughton, MA) according to the manufacturer’s protocol. Samples were read at 450-nm wavelength using a SpectraMax 250 microplate reader.

Alkaline phosphatase activity, cells were extracted in 1% Triton X-100 in HEPES buffer, pH 7.4, and then sonicated and centrifuged at 14,000 g for 4 min. Supernatants were stored at −80°C until analysis. A reaction buffer, pH 7.4, composed of 100 mM glycine buffer, 1 mM MgCl2, and 1 mM ZnCl2 in distilled water was added with 60 mM p-nitrophenyl phosphate (Sigma) to the cell supernatants, and then alkaline phosphatase was measured spectrophotometrically (SpectraMax 250 microplate reader) at 405-nm wavelength. Osteocalcin levels in the medium were measured after 24-h serum starvation using a commercial enzyme immunoassay kit (Biomedica, Stoughton, MA) according to the manufacturer’s protocol. Samples were read at 450-nm wavelength using a SpectraMax 250 microplate reader.

Microtubule and nuclear staining. Staining and imaging of differentiating cells. Microtubule and nuclear staining were obtained using a fluorescent microscope (Zeiss LSM 510) with differential interference contrast (DIC) microscope to illustrate the formation of nodules with compartmentalized morphology.

Immunocytochemistry. Within 5 min of stopping the centrifuge, cells for microtubule and nuclear staining were washed with Dulbecco’s PBS (GIBCO-BRL/Invitrogen, Grand Island, NY) at 37°C, fixed in ice-cold methanol for 5 min, washed with PBS, and incubated for 30 min in a blocking solution containing 5% bovine serum albumin (Sigma), 0.1% Tween 20 (Fisher Scientific, Fair Lawn, NJ), 2% goat serum (Jackson ImmunoResearch, West Grove, PA), and PBS. Cells were incubated in primary antibody (1:200 dilution mouse monoclonal anti-chicken α-tubulin, clone DM 1A; Sigma), diluted in the blocking solution for 1 h at room temperature, then washed with a solution containing 0.1% Tween 20 (Fisher Scientific), 5% bovine serum albumin (Sigma), and PBS, and then incubated for 20 min at room temperature with Texas Red goat anti-mouse secondary antibody (Jackson ImmunoResearch) diluted 1:200 in blocking solution with 0.5 mM Sytox Green nuclear stain (Molecular Probes, Eugene, OR). Finally, slides were washed with blocking solution and rinsed with distilled water. Coverslips were placed on slides with Aqua PolyMount (Polysciences, Warrington, PA) and sealed with nail polish.

Microtubule and nuclear morphology. Serial optical images of the microtubule cytoskeleton and cell nuclei were obtained using a confocal laser scanning microscope equipped with DIC optics, a ×63 magnification, 1.25 numerical aperture oil-immersion lens objective, and a 30-nW argon/krypton laser (LSM 510; Carl Zeiss, Thornwood, NY). Pinhole sizes and photomultipliers were set to produce the clearest possible image without saturating the signal. After the conditions of image acquisition were optimized for the 1-g control cells, images of hypergravity-stimulated and 1-g control cells were collected using identical settings. Several techniques were used to identify morphological changes, including characterization of optical slices through the midplane of the cell, construction of image galleries containing each serial slice in an image stack, and projection of all slices into one view. To analyze microtubule network height, image z-stacks were acquired and post-image analysis software was used to draw a horizontal line in the x-y plane across the center of the image field. The profile function was used to provide signal intensity for each pixel along the line, yielding a graphed plot of intensity vs. distance. The image stacks were viewed from the top slice to the bottom slice.
the graphical intensity plot changes were observed, and all images with an intensity value >50 anywhere along the line were included in the calculation of height. The intensity threshold of 50 was determined in tests using fluorescent beads of known size to best represent actual signal and not background noise. The resulting number of slices was multiplied by the slice thickness (0.5 μm) to yield the microtubule network and nuclear height. Microtubule network height of the multicell layer in differentiating cultures was measured from the top to the bottom image in the confocal image stack to provide an indication of the overall height of the nodular and internodular regions. The number of nuclei in a z-axis orthogonal view was counted to provide an indication of the number of cell layers.

Statistics. Data are representative of three to six separate experiments performed with four individual culture wells per condition. PGE2 analyses were performed in duplicate. Cell number and alkaline phosphatase were performed on four samples and osteocalcin on six samples. Eight microscopic fields per condition were evaluated for microtubule morphology, network height, and alizarin red staining. Values are expressed as means ± SE. Statistical evaluation was performed using StatView version 5.0.1 software (SAS Institute, Cary, NC). Differences were compared using ANOVA with a significance level of 0.05. P < 0.05 was accepted as significant, with P values corrected by applying the Bonferroni adjustment to Fisher’s protected least-significant difference post hoc analysis.

RESULTS

1-FDC provides a thermal and vibration environment comparable to that of a control incubator. Thermal control tests of 1-FDC (Fig. 1) showed that centrifugation for 3 h before the experiment stabilized the centrifuge temperature at 37 ± 0.2°C. By placing samples on prewarmed platforms, closing the centrifuge lid within 30 s, and then resetting the rotation rate, temperature within the centrifuge decreased transiently by 0.5°C and then returned to 37°C within 12 min. This transient was similar to that which occurred when opening and closing the door of the control incubator. The CO2 levels remained steady at 5 ± 0.1%, with a 1% transient decrease correspond ing to opening and then closing the centrifuge; CO2 levels recovered to 5% within 12 min. Evaporation from the culture wells was assessed to validate humidity control. After a 3-h spin at 238 rpm (10 g), the amount of medium was reduced by <5%.

Tests to characterize the centrifuge vibration environment in the resultant gravity vector direction were performed. When the centrifuge was spinning at 238 rpm (10 g), a peak value of $10^{-4}$ g, rms was measured at a frequency of 4 Hz, with additional peaks of $2\times 10^{-4}$ g from ~4 to 85 Hz. The majority of the vibrations were measured in the range of $10^{-6}$ to $10^{-5}$ g, rms from 0 to 100 Hz, which was 1% × $10^{-5}$ to 1% × $10^{-4}$ that of a 10-g hypergravity stimulus. In comparison, the control incubator generated vibrations of $10^{-6}$ to $10^{-5}$ g, rms from 0 to 100 Hz, which was similar to the centrifuge, with a resonant peak of $10^{-2}$ g, rms at 17 Hz. Thus the centrifuge and control incubator provided comparable vibration environments.

Hypergravity increased PGE2 release and decreased microtubule network height in a dose-dependent manner. We initially assessed the influence of a 10-g hypergravity stimulus on PGE2 release; overall cell, microtubule cytoskeleton, and nuclear morphologies; microtubule network height; and nuclear height. We then tested stimuli <10 g and >10 g. Confluent osteoblasts (d3) subjected to 10 g for 3 h released 490 pg/ml PGE2 compared with 190 pg/ml released by 1-g control cells, representing a 2.5-fold increase (Fig. 2). A 2.5-g stimulus did not result in detectable PGE2 release compared with 1-g controls (data not shown). A 5-g stimulus resulted in a 2.5-fold increase in PGE2 release compared with controls; this change was not significantly different from the effects of a 10-g stimulus. Hypergravity stimuli >10 g applied for 3 h further increased PGE2 release. A 15-g stimulus resulted in a 3.4-fold increase, and a 50-g stimulus resulted in a 5.3-fold increase, compared with controls. The PGE2 release due to 50 g was significantly different from that at 5, 10, and 15 g. The difference between 10 and 15 g was significant, but the difference between 5 and 15 g was not.

The morphology of osteoblasts subjected to 10-g stimulation for 3 h was similar to that of controls (Fig. 3, A–D). A healthy, densely confluent cell layer was observed in both 1- and 10-g cultures. Nuclear morphology in an image slice through the midplane of the cell (Fig. 3C) indicated no evidence of mechanical damage in 10-g cultures, and the overall cell morphology was indistinguishable from controls (Fig. 3A). Microtubules in the same confocal slice exhibited interconnected, radially emanating network structures in 1 g (Fig. 3B) and 10 g (Fig. 3D) conditions with similar architecture. Hypergravity caused a small increase in the intensity of the labeled micro-

**Fig. 1.** 1-foot diameter centrifuge (1-FDC). Osteoblasts were exposed to hypergravity using the 1-FDC at the National Aeronautics and Space Administration Ames Research Center for Gravitational Biology Research (see http://lifesci.arc.nasa.gov/CGBR/1_ft.html). A tabletop centrifuge with four swinging buckets was modified to provide lower rotation rates (45–1,000 rpm yielding 1.4–180 g) and environmental monitoring. The centrifuge was integrated with an incubator to control temperature, humidity, and CO2. Fans circulated air through insulated ducts, and water traps collected condensation. An identical incubator was used for stationary controls. Environmental data from the control incubator and the integrated centrifuge-incubator system were collected using a data acquisition system.
tubules, but the difference in fluorescence intensity was not significant (data not shown). Exposure to 10-g hypergravity decreased the microtubule network height by 12% (Fig. 4), but nuclear height was not different (data not shown). No differences were observed between cultures in the control incubator and cultures in the 1-FDC maintained without rotation at 1 g, showing that rotation was responsible for the changes induced by centrifugation.

Increasing levels of hypergravity exerted no detectable effects on cellular, microtubule network, or nuclear morphologies. In all cases, the cells appeared healthy, nuclei were intact, and radially emanating microtubule networks were observed.

Microtubule network height did not change at 2.5-g stimulation (data not shown), but decreased from 5 g (7% decrease) to 50 g (26% decrease) (Fig. 4). The 50-g stimulation resulted in a significantly reduced microtubule network height compared with the 1-g control and the 5-g stimulation. Nuclear height was unaffected at any hypergravity dose tested (data not shown).

**Time course of response.** As a control for possible transient effects of acceleration and deceleration, cells were accelerated to 10 g and then immediately decelerated to 1 g and compared with the 1-g controls. No differences were noted (Fig. 5). Increasing the duration of the 10-g stimulus to 10 min resulted in a 1.5-fold increase in the amount of PGE2 released into the medium. Further increases were observed as the 10-g stimulus was lengthened to 1 h (1.5-fold), 3 h (2.8-fold), and 6 h (4.8-fold); all increases were significant. The rate of PGE2 release was calculated to determine whether the rate depended on the duration of the stimulus (see MATERIALS AND METHODS). Exposure to hypergravity for 10 min caused a 10-fold increase in the calculated rate of PGE2 release compared with the 1-g control (Table 1). The 1-, 3-, and 6-h exposures resulted in 2.6-, 2.9-, and 4.3-fold increases in the rate of PGE2 release, respectively, compared with the 1-g control. Changing the duration of 10-g hypergravity exposure from 10 min to 6 h did not affect the overall appearance of the cells or nuclei. Only the 3-h exposure resulted in a significant reduction in microtubule network height, while shorter durations slightly but not significantly reduced network height (data not shown). Nuclear height was not affected by hypergravity exposure at any duration tested (data not shown).

**Influence of gravity vector direction.** D3 cells were centrifuged for 3 h at 10 g in a flat or side orientation, either
Confluent osteoblasts \((d3)\) were subjected to 10-g \(g\) hypergravity from 5 to 50 g for 3 h. Microtubule network height decreased as hypergravity increased. \(*P < 0.05\), significant difference compared with 1-g controls. \(**P < 0.001\), significant difference compared with corresponding 1-g controls.

To calculate the amount of shear stress applied to the osteoblasts when they were oriented on their side, the density of ROS 17/2.8 osteoblast-like cells was measured as described in MATERIALS AND METHODS. The ROS medium density was determined to be 0.99 g/ml, and medium kinematic viscosity was 1.4 \(10^{-2}\) m/s. The average cell diameter was 19 \(\mu\)m, the cell density was 1.04 g/cm\(^3\), and the area near the growth substrate was 730 \(\mu\)m\(^2\). From these measured values, cell volume and mass were calculated as \(3.6 \times 10^{-15}\) m\(^3\) and \(3.73 \times 10^{-15}\) g, respectively. On the basis of these values, 10-g stimulation resulted in shear stress of 0.5 Pa (5 dyn/cm\(^2\)).

Characterization of osteoblast differentiation. Osteoblast differentiation in a 1-g environment was characterized by evaluating the overall osteoblast culture morphology as shown using DIC imaging (Fig. 6, A–D) and alizarin red staining of bone nodules (Fig. 6, E and F), alkaline phosphatase activity, osteocalcin content in the media, microtubule network height, and PGE\(_2\) release. Cells were grown for 3–4 days to confluence (Fig. 6A), and the medium was supplemented with AA and \(\beta\)-GP to induce differentiation. Cells formed a uniform layer, and nuclear and microtubule network morphologies were as shown in Fig. 3. By \(d6\), discrete regions of cuboidal cells (prenodules) appeared within the confluent layer (Fig. 6B, arrow) and multilayered nuclei and microtubule networks were observed (data not shown). By \(d9\), cells had multilayered further and produced abundant extracellular matrix (Fig. 6C; arrow) and small alizarin red-stained mineralized nodules (Fig. 6E; arrow). By \(d19\), mature mineralized nodules formed as shown using DIC (Fig. 6D, arrows) and alizarin red staining (Fig. 6F, arrows) and were surrounded by unmineralized internodular regions. Cell number, alkaline phosphatase activity, and osteocalcin increased as the cells differentiated (Table 2).

Quantification of the multicell microtubule network height in a mature, multilayered culture provides an indication of the overall height of nodular and internodular regions. Confocal images were analyzed with the same method used for confluent cell layers whereby the height was measured from the top to the bottom image in the confocal image stack. The number of nuclei in a \(z\)-axis orthogonal view were counted to provide an estimate of the number of cell layers. The number of cell layers within nodules appeared to increase from one layer on \(d3\), one to two layers on \(d6\), two layers on \(d9\), and three to four layers on \(d19\). The nodule height increased as the culture progressively differentiated (Fig. 7) \((P < 0.001)\), due partially to increased numbers of cell layers but also to increased spacing between nuclei by \(d19\). By \(d9\), the height of the internodular regions was significantly less (7 \(\mu\)m) than that of the nodules (17 \(\mu\)m) \((P < 0.001)\), but by \(d19\), the height of both the nodular and internodular regions had increased to 25 \(\mu\)m. In the internodular regions, cell layer number increased from one layer on \(d3\) to three layers on \(d19\).

In contrast to nodular and internodular height, PGE\(_2\) released per 10\(^6\) cells in a 1-g environment did not appear to change significantly with differentiation through \(d9\) (Fig. 8). By \(d19\) in culture, PGE\(_2\) release was not detectable (data not shown).

### Table 1. Dependence of rate of hypergravity-induced PGE\(_2\) release on hypergravity duration

<table>
<thead>
<tr>
<th>Gravity Condition</th>
<th>Rate of PGE(_2) Release, pg/ml (\cdot)min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 g</td>
<td>1.3 1.7 0.9 0.6</td>
</tr>
<tr>
<td>10 g</td>
<td>13.0 4.4 2.6 2.6</td>
</tr>
</tbody>
</table>

The rate of prostaglandin E\(_2\) (PGE\(_2\)) released as a result of increasing exposure to hypergravity was calculated as described in MATERIALS AND METHODS. A 10-min stimulus of 10-g hypergravity resulted in the highest rate of PGE\(_2\) release.
Effects of osteoblast differentiation on responses to hypergravity. We asked whether the response to a 10- or 50-g hypergravity stimulus was associated with the stage of differentiation. A 10-g stimulus for 3 h caused a fivefold increase and a 50-g stimulus for 3 h caused a sixfold increase in PGE₂ released per 10⁶ cells from d₆ cultures compared with the 1-g control (P < 0.001), and the difference in PGE₂ release between 10 g and 50 g was not significant. Neither 10 g nor 50 g resulted in increased PGE₂ released per 10⁶ cells from d₉ cultures (Fig. 8). Hypergravity did not induce d₁₉ cultures to release PGE₂ (data not shown).

In the latter stages of differentiation (d₉ and d₁₉), microtubule network height in nodular and internodular regions did not change consistently as a result of either 10 g or 50 g applied for 3 h (data not shown). In d₆ cultures, 10 g resulted in a 10% decrease in internodular and nodular height, but this difference was not statistically significant (data not shown). Nuclear height was insensitive to hypergravity at all days in culture,
and there was no evidence of apoptosis or other indications of poor cell health, regardless of the gravity level examined (data not shown).

**DISCUSSION**

As a first part of this study, we characterized the properties of the 1-FDC and found that the centrifuge provides a thermal and vibration environment comparable to that of a control incubator. The results revealed that both PGE$_2$ release and microtubule network height in confluent primary osteoblasts were sensitive to hypergravity in a dose- and time-dependent manner and that hypergravity-induced PGE$_2$ release appeared blunted in mature osteoblasts. Hypergravity applied to confluent (d3) cultures at 10 g for 3 h resulted in increased PGE$_2$ release, decreased microtubule network height, and no major changes in overall morphology or viability. When the magnitude of the hypergravity stimulus was varied from 5 to 50 g, PGE$_2$ release rose without reaching a plateau and microtubule network height gradually declined. PGE$_2$ release in response to hypergravity was time dependent but was not influenced by the orientation of the gravity vector. When primary osteoblasts at different stages of differentiation were exposed to hypergravity, PGE$_2$ release increased through the initial stages of matrix deposition when nodules first formed (d6). In contrast to immature cultures, cultures with mineralized matrix failed to show sensitivity to hypergravity stimulation. Taken together, these results indicate that changes in the magnitude of the gravity vector regulate cytoskeletal structure as indicated by microtubule network height, as well as function as indicated by release of the paracrine signaling factor PGE$_2$. Furthermore, our results indicate that immature osteoblasts are more sensitive than more mature cells to a hypergravity stimulus.

Validation of hypergravity as a model for studying the influence of the gravity vector. To develop a valid tool to study hypergravity, the physical environment of the centrifuge must replicate the environment of the control cultures. Furthermore, the impact of various physical factors that may influence cellular responses to centrifugation other than hypergravity per se should be considered. To characterize the physical environment, we ensured that the temperature, CO$_2$, and humidity conditions in the centrifuge (1-FDC) and control incubator were similar. Next, we measured vibration in the centrifuge because low-frequency vibrations are known to regulate osteoblast activities (40). The 1-FDC displayed a low level of broad frequency vibration (10$^{-6}$ to 10$^{-3}$ g; rms; peak 10$^{-4}$ g, rms at 4 Hz) in the direction of the resultant gravity vector; these values are well below the magnitude of a 10-g hypergravity stimulus (1% $\times$ 10$^{-5}$ to 1% $\times$ 10$^{-3}$). Furthermore, there were no changes in PGE$_2$ release or microtubule network height in cultures maintained in the 1-FDC at 1 g (without rotation) compared with cultures maintained in the control incubator. On the basis of these results, we conclude that the 1-FDC and control incubators provide comparable physical environments for cell growth.

To evaluate the various physical factors contributing to the centrifuge environment, we calculated the gravity gradient, coriolis force, and inertial shear contributions to cell cultures for the 1-FDC in producing acceleration of 10 g (238 rpm). Objects on a centrifuge are exposed to a gravity gradient. Given the measured cell height of $\sim$4 $\mu$m, the difference in acceleration between the two opposite cell surfaces is 2.6% $\times$ 10$^{-4}$ of 10 g. A centrifuge also causes motile cells to experi-
ence a coriolis force. Coriolis acceleration is defined as $a_c = 2 \nu \omega$, where $\nu$ is the radial velocity of a motile cell and $\omega$ is the angular velocity of the centrifuge. Assuming an average osteoblast motility of 10 $\mu$m/h (13) on the 1-FDC at 10 g, the coriolis acceleration is only $1.41 \times 10^{-8}$ g, or $1.41 \times 10^{-7}$ of 10 g. Finally, because the culture surface is flat, the gravity vector is not uniform. This results in a net acceleration, termed inertial shear, toward the edges of the platform (46). From the center of the platform to the edge, the inertial shear varies from 0 to 3% of 10 g (given a platform width of 9 cm). By using the central four wells of the eight-well chamber slide (2 cm total width) and placing the slide in the middle of the platform, the inertial shear for these experiments was limited to 0.67% of 10 g. These gravity variations due to centrifugation in this apparatus are summarized in Table 3. While the inertial shear is the largest artifact resulting from using the 1-FDC to simulate hypergravity, it still represents only a small contribution to the gravity levels used in these studies (2.5–50 g).

The final question we asked was what levels of hypergravity stimulation are likely to be physiologically relevant for cells that reside in bone. Gravity generates hydrostatic pressure due to the column of medium above the cultures when oriented with the gravity vector orthogonal to the cell growth substrate. Hydrostatic pressure is calculated as $P = \rho gh$, where $\rho$ is the density of the medium, $g$ is the acceleration of gravity, and $h$ is the height of the column of medium. For our cell culture system, the hydrostatic pressure at the substrate was calculated to be 1.2 kPa at 10 g and 6 kPa at 50 g. These pressure levels are comparable to the intraosseous and intramedullary pressures applied to osteoblasts growing on trabeculae in vivo, which are 1–5 kPa in dogs (4) and 2 kPa in rats (6). Thus the hydrostatic pressure levels produced by centrifugation from 10 to 50 g are in the physiological range for the osseous environment.

Hypergravity may cause increased strain due to cell deformation, in addition to increased hydrostatic pressure. To estimate the strain due to gravity acting on an osteoblast, Hatton et al. (18) modeled an osteoblast as a homogeneous elastic disk with the material properties of a chondrocyte. They concluded that hypergravity levels of 4–30 g resulted in 40–300 microstrains, respectively, similar to those observed in humanibia during light exercise (8). To assess the combined effects of hydrostatic pressure and strain on an osteoblast with a heavier nucleus and a discrete cytoskeleton, we developed a cell model that included a plasma membrane, a nucleus 40% heavier than the surrounding cytoplasm, and actin and microtubule cytoskeletal networks with material properties derived from the literature (43, 44). Results indicated that hypergravity levels of 10 g resulted in a 5% reduction in cell height, and strains varied by several orders of magnitude, depending on location within the cell (e.g., outer plasma membrane vs. microtubules). On the basis of these analyses, we concluded that exposing osteoblasts grown on a substrate oriented perpendicular to the gravity vector to centrifugation from 2.5 to 50 g resulted in mechanical strains within the physiological range.

Cells oriented with the growth substrate parallel to the gravity vector are subject to shear stress due to gravity acting on the cell mass. With the use of measured values of ROS 17/2.8 osteoblast-like cell mass and area near the growth substrate, a 10-g stimulus resulted in a shear stress of 0.5 Pa (5 dyn/cm²). This value of shear stress is similar to shear induced by fluid flow, which has been shown to act as a mechanical stimulus to osteoblasts in vitro (3, 23, 39, 40), and is predicted to be applied to osteocytes in vivo (51). Therefore, cells in this orientation experienced a shear stress in the physiological range.

Response of immature osteoblasts to hypergravity. With the hypergravity model established, we investigated the characteristics of hypergravity-induced changes in microtubules and release of the paracrine signaling factor PGE$_2$, which is a critical component of the anabolic response of bone to mechanical loads (11). Exposure of confluent, immature osteoblasts (d3) to hypergravity ranging from 5 to 50 g for 3 h resulted in a 2.5- to 5.3-fold increase in PGE$_2$ release compared with 1-g controls. These results are consistent with the finding that a short, intensive pulse (5 min, 187 g) of centrifugation triggers PGE$_2$ release from MC3T3-E1 osteoblasts (14). In our study, changes in PGE$_2$ release were not observed after a 2.5-g stimulus, suggesting either insensitivity to small gravity changes or that PGE$_2$ release is not a sensitive cellular response. The dose-response curve from 5 to 50 g indicates that 5 g was the minimum effective dose for PGE$_2$ release, and this release increased to 50 g. Because the differences in PGE$_2$ release between 5-, 10-, and 15-g stimuli were small, it may be that the response to gravity levels up to 15 g are the first phase of a response and that the response to 50 g represents a second phase. This hypothesis is consistent with osteoblast responses to mechanical deformation of the growth substrate; studies have shown biphasic responses to increasing levels of strain (30).

Centrifugation at increasing gravity levels also caused a gradual decline in microtubule network height to a 26% decrease in 50-g cultures relative to 1-g controls. These results show that there is a correlation between PGE$_2$ release and microtubule network height as the magnitude of the hypergravity stimulus is raised. When ROS 17/2.8 osteoblastic cells were subjected to alternating hypergravity and microgravity in parabolic flight, a positive correlation between cell area and intracellular PGE$_2$ levels was demonstrated when all gravity levels were considered (17), suggesting that rapid changes in the direction of the gravity vector may affect cell shape. However, we found that acceleration followed by immediate deceleration failed to exert the same effects on microtubule network height and PGE$_2$ release as exposure to a continuous hypergravity stimulus.

Although the microtubule network height decreased with increasing gravity levels, the nuclear height did not appear to change. This suggests that the nucleus did not displace downward toward the substrate in the increased gravity field, al-

### Table 3. Mechanical components of loading by hypergravity

<table>
<thead>
<tr>
<th>Gravity Variations in 1-FDC (238 rpm, 10 g), %10 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrational acceleration</td>
</tr>
<tr>
<td>Gravity gradient from top to bottom of cell layer</td>
</tr>
<tr>
<td>Coriolis</td>
</tr>
<tr>
<td>Inertial shear</td>
</tr>
</tbody>
</table>

Variations in the gravity field in the 1-foot diameter centrifuge (1-FDC) were estimated while being spun at 238 rpm to generate 10-g hypergravity. Vibration was measured, and the gravity gradient, coriolis, and inertial shear contributions were calculated.
though repositioning of the nucleus may have been below the limits of detection.

PGE$_2$ release and microtubule network height demonstrated similar dose dependency, but the time courses of these responses appeared to differ. PGE$_2$ release increased as the duration of the hypergravity stimulus was lengthened from 10 min (1.5-fold) to 6 h (4.8-fold); yet only a 3-h duration resulted in a significant reduction in microtubule network height. By 3 h, the microtubule network may have adapted to form a more stable configuration.

Hypergravity stimulated PGE$_2$ release whether the cultures were oriented parallel (side) or perpendicular (flat) relative to the gravity vector. Because hydrostatic pressure acts in all directions, the cultures in the side orientation still experienced pressure. The strains applied to cultures in the side orientation due to the shear stress acting on the cell’s mass were different from the strains applied to cultures in the flat orientation, owing to the compressive load in the flat orientation. The PGE$_2$ release could be attributed to the influence of hydrostatic pressure in both cases.

**Response of differentiating osteoblasts to hypergravity.** The hypergravity-induced release of PGE$_2$ and reduction in microtubule network height differed depending on the duration of cell culture. We confirmed that differentiation in vitro recapitulates the major features of osteoblast differentiation in vivo as shown in other studies (5, 16, 29, 33). Treatment with AA and β-GP in the continuous presence of 10% serum caused progressive changes in characteristic features of the mature osteoblast, including acquisition of a cuboidal morphology, increased alkaline phosphatase activity, production of a collagenous extracellular matrix that mineralized, and osteocalcin production.

The PGE$_2$ released in control cultures at 1 g was relatively constant from confluence (d3) through early nodular mineralization (d9) but fell to undetectable levels at latter stages of culture when nodules were mineralized (d19). These results are consistent with the decline in PGE$_2$ during differentiation of adult rat calvarial osteoblasts reported by Fujieda et al. (15).

We found that hypergravity increased PGE$_2$ release and reduced microtubule height in confluent (d3) and early noduliforming (d6) cultures (3 h at 10 or 50 g), demonstrating sensitivity to hypergravity through the initiation of nodule formation. In contrast, hypergravity stimuli failed to induce PGE$_2$ release or microtubule network height changes in more mature cultures (d9–d19), demonstrating a possible decline in sensitivity during later stages of nodule maturation and mineralization.

Given the conditions of cell growth used in this study, variables other than differentiation per se also may contribute to the reduced gravity sensitivity observed at the later time points in culture (45). Continuous growth in relatively high concentrations of fetal calf serum (10%), together with supplementation with AA and β-GP is currently the standard condition used for growth and differentiation of rat primary osteoblasts (5, 16, 24, 29, 33). However, other potentially important factors that may contribute to the changes observed over time in this study include cellular aging; sustained exposure to high concentrations of growth factors, hormones, and other ill-defined serum factors; and/or altered cell-cell interactions resulting from high cell density. To control for additional proliferation in maturing cultures, PGE$_2$ release was corrected to cell number. Another possible explanation for the reduced sensitivity to hypergravity that we observed in mature cultures is that the abundant extracellular matrix and cell multilayering, which are present only in mature cultures, blunted transmission of gravity loads to the osteoblasts. Alternatively, the cyclooxygenase responsible for PGE$_2$ production in response to the hypergravity stimulus may be present in lower levels in our mature cultures.

In any event, our results show that the sensitivity to hypergravity appears highest at less mature stages of osteoblast differentiation, when cells are confluent but are not yet producing a matrix that is mineralized. Miwa et al. (28) suggested that hypergravity stimulates proliferation in early cultures and that PGE$_2$ mediates this response. Consistent with our findings, human fetal osteoblasts lose their sensitivity to mechanical stretch at late stages of differentiation (45).

In conclusion, we have shown that a continuous hypergravity stimulus induced PGE$_2$ release and reduced the height of the microtubule network in primary fetal rat osteoblasts. These responses depended on the magnitude and duration of the stimulus. Immature osteoblasts appeared most sensitive to changes in gravity loading. Our results demonstrate the utility of centrifugation as an experimental tool to study the influence of changes in the gravity vector on cell structure and function.

**ACKNOWLEDGMENTS**

We thank Soha Motlagh and Indroneal Banerjee for technical assistance. We thank the National Aeronautics and Space Administration (NASA) Ames Research Center for Gravitational Biology Research support team, including Tianna Shaw, Duncan Atchison, Anthony Purcell, and Ed Houston, for their help with 1-FDC modifications and testing and Marty Hasha for assistance with vibration testing. We thank Robert Majeska for the gracious gift of ROS 17/2.8 cells and Beckman Coulter for the loan of the QuickScan dispersion analyzer. We also thank Sigrid Reinsch and Eduardo Almeida for helpful advice during the course of the study and Wenonah Vercoultre and Emily Holton for critical reading of the manuscript.

**GRANTS**

This work was supported by NASA Grants NAGS-5-6734 and ARC DDF-96-01.

**REFERENCES**


