Journal of Cellular Biochemistry 102:483–495 (2007)

The Effect of Simulated Microgravity on Osteoblasts Is Independent of the Induction of Apoptosis

M.A. Bucaro,[1] A.M. Zahm,[1] M.V. Risbud,[1] P.S. Ayyaswamy,[2] K. Mukundakrishnan,[2] M.J. Steinbeck,[1] I.M. Shapiro,[1] and C.S. Adams[1]*

[1]Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania
[2]School of Engineering and Applied Sciences University of Pennsylvania, Philadelphia, Pennsylvania

Abstract Bone loss during spaceflight has been attributed, in part, to a reduction in osteoblast number, altered gene expression, and an increase in cell death. To test the hypothesis that microgravity induces osteoblast apoptosis and suppresses the mature phenotype, we created a novel system to simulate spaceflight microgravity combining control and experimental cells within the same in vitro environment. Cells were encapsulated into two types of alginate carriers: non-rotationally stabilized (simulated microgravity) and rotationally stabilized (normal gravity). Using these specialized carriers, we were able to culture MC3T3-E1 osteoblast-like cells for 1–14 days in simulated microgravity and normal gravity in the same rotating wall vessel (RWV). The viability of cells was not affected by simulated microgravity, nor was the reductive reserve. To determine if simulated microgravity sensitized the osteoblasts to apoptogens, cells were challenged with staurosporine or sodium nitroprusside and the cell death was measured. Simulated microgravity did not alter the sensitivity of C3H10T-1/2 stem cells, MC3T3-E1 osteoblast-like cells, or MLO-A5 osteocyte-like cells to the action of these agents. RT-PCR analysis indicated that MC3T3-E1 osteoblasts maintained expression of RUNX2, osteocalcin, and collagen type I, but alkaline phosphatase expression was decreased in cells subjected to simulated microgravity for 5 days. We conclude that osteoblast apoptosis is not induced by vector-averaged gravity, thus suggesting that microgravity does not directly induce osteoblast death. J. Cell. Biochem. 102: 483–495, 2007.

Key words: microgravity; osteoblast; apoptosis; osteopenia; alginate

A marked and potentially debilitating reduction in bone mass is common after extended time in space, a condition known as spaceflight osteopenia [Mack et al., 1967; Vose, 1974; Atkov, 1992; Oganov et al., 1992; Collet et al., 1997]. Based on studies of other osteopenic states, it is probable that spaceflight osteopenia reflects both an increase in bone resorption by osteoclasts and a decrease in bone formation by osteoblasts [Caillot-Augusseau et al., 1998]. Evaluations of astronauts have shown that markers of bone formation [procollagen type I C-terminal fragment and bone alkaline phosphatase] are decreased during space flight [Collet et al., 1997; Caillot-Augusseau et al., 1998]. Spaceflight studies have also revealed that isolated osteoblasts are directly sensitive to changes in gravity [Guignandon et al., 1995; Hughes-Fulford and Lewis, 1996; Carmeliet et al., 1997, 1998; Guignandon et al., 1997; Hughes-Fulford et al., 1998; Hughes-Fulford and Gilbertson, 1999; Kumei et al., 1999; Sato et al., 1999; Harris et al., 2000; Landis et al., 2000; Hughes-Fulford et al., 2006]. Osteoblasts cultured in microgravity are generally characterized by a contracted morphology, a decrease in cell number, a reduction in osteoblast-specific gene expression, and a lowered response to anabolic stimuli, relative to unit gravity controls [Hughes-Fulford, 2002]. These observations have lent considerable support to the hypothesis that microgravity causes bone loss during spaceflight through a direct cellular effect on the osteoblast. We and others have proposed that bone loss may be a consequence of osteoblast viability caused by the activation of apoptosis [Bucaro et al., 2004; Mukundakrishnan et al., 2004].

*Correspondence to: C.S. Adams, Department of Orthopaedic Surgery, Thomas Jefferson University, 1015 Walnut St., 501 Curtis Bldg. Philadelphia, PA 19107.
E-mail: christopher.adams@jefferson.edu
Received 20 July 2006; Accepted 25 January 2007
DOI 10.1002/jcb.21310

Since the opportunities to test the apoptosis hypothesis in spaceflight are rare, clinostats, such as the NASA-engineered high aspect ratio vessel (HARV) or other rotating wall vessels (RWV) [Dai et al., 2004] have been used to simulate microgravity [Kunisada et al., 1997; Sato et al., 1999; Sarkar et al., 2000a,b; Granet et al., 2001, 2002; Rucci et al., 2002; Yuge et al., 2003; Nakamura et al., 2003b; Patel et al., 2007]. By dynamically changing the orientation of cells with respect to gravity, clinostats time-averaged the gravitational field to near zero over each revolution (vector-averaged gravity), thus effectively negating the influence of gravitational sedimentation [Sievers and Hejnowicz, 1992; Klaus, 2001]. These studies have generated considerable confusion and conflicting results. For example, Sarkar et al. [2000a,b] and Rucci et al. [2002] reported that viability was substantially reduced in osteoblasts under clinorotation for 24 h, and furthermore, they reported that loss of viability was due to increased apoptosis. In contrast, our laboratory [Bucaro et al., 2004] has, in agreement with Nakamura et al. [2003a], reported that following several days of clinorotation, cell viability was unaffected. However, we did observe that cells subjected to clinorotation were compromised metabolically and were more susceptible to apoptogens [Bucaro et al., 2004]. A significant problem in ground-based studies is that each system subjects cells to stimuli that could confound the effects of simulated microgravity, most importantly, fluid flow induced shear stresses, vibration, and convective mixing. The divergent results reported highlight the need to control for these extraneous factors.

We have developed a new method to evaluate the effects of vector-averaged gravity on osteoblasts that addresses the concerns stated above. Herein, we describe a novel system that allows control osteoblasts to be cultured in normal (unit) gravity within the same HARV as cells experiencing simulated microgravity (vector-averaged gravity). For this purpose, control cells are contained within rotationally stabilized carriers that do not spin in the HARV. These cells are maintained in normal gravity conditions, whereas cells cultured in non-rotationally stabilized carriers, that freely spin in the HARV, experience vector-averaged gravity. Thus, it is possible to control for the extraneous factors, inherent to HARV culture, which might predispose osteoblasts to apoptosis. Using this system, we tested the hypothesis that vector-averaged gravity increases the induction of osteoblast death. We show that vector-averaged gravity in the absence of extraneous factors did not affect the viability of the cells nor increase their sensitivity to common apoptogens.

MATERIALS AND METHODS

System Design

We fabricated two types of alginate beads to support osteoblast culture in the HARV: rotationally stabilized and non-rotationally stabilized carriers, designated polar and non-polar carriers, respectively. The non-polar carriers were free to spin about their own axes in the HARV while polar carriers remain in a constant orientation. The centers of buoyancy and gravity coincide in the non-polar carriers, giving them no preferred orientation (Fig. 1A). As the HARV rotates, non-polar carriers spin on their own axis (Fig. 1B) due to the near-solid body motion of the medium. Polar carriers are created with a buoyant pole that imparts rotational stability. These carriers have a preferred orientation due to the separation between the centers of gravity and buoyancy (Fig. 1C) and, therefore, overcome the forces exerted by medium and do not spin about their own axes (Fig. 1D). Accordingly, as the HARV rotates, cells in non-polar carriers are subjected to vector-averaged gravity while cells in polar carriers remain in normal gravity conditions.

Generation of Alginate Carriers

Alginate carriers were created using a modified electrostatic generator based on a previously described method [Klokk and Melvik, 2002]. The device included a tapered stainless steel needle, a glass tubing port, a gelling bath, a variable power supply, and a syringe pump [Klokk and Melvik, 2002]. A schematic of the generator is shown in Figure 1E. MC3T3-E1 cells were suspended in DMEM supplemented with 10% fetal bovine serum, and mixed with alginate (Ultra Pure MVG isolated from laminaria hyperborean) (NovaMatrix-FMC BioPolymer, Philadelphia, PA) to produce a 1% alginate (w/v) solution containing 2×10^6 cells/ml. Droplets were formed by extruding the alginate-cell suspension through the 18-gauge needle at a rate of 100 ml/h. Upon entry into the cross-linking bath (25 mM $CaCl_2$, 112 mM NaCl), the alginate droplets polymerized,

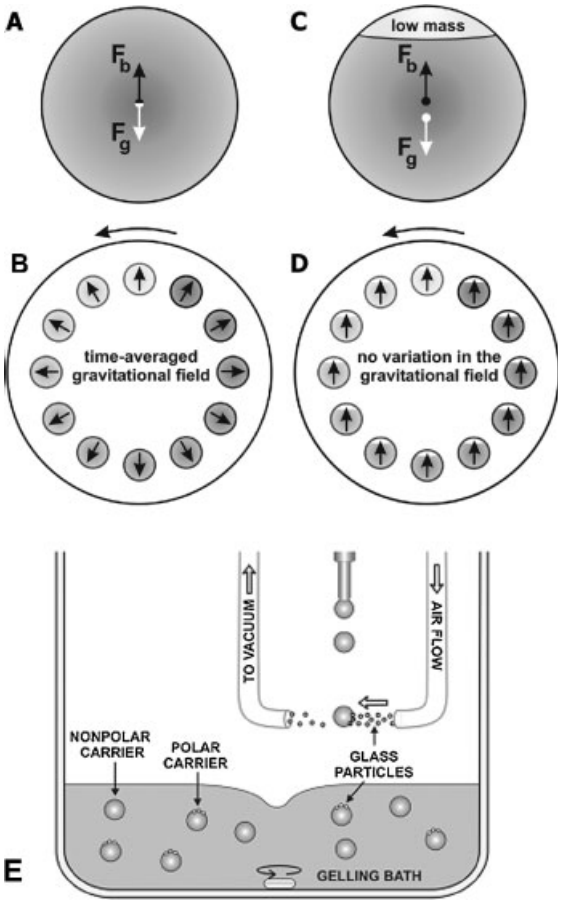

Fig. 1. A–D: Schematic of forces that control the spin of alginate carriers in the rotating HARV. The carriers are designed so that the forces of gravity (Fg) and buoyancy (Fb) are nearly balanced. As such, the carriers move in near solid body rotation with the fluid in the HARV. In a radially symmetric carrier, the center of buoyancy coincides with the center of gravity (A). Such non-polar carriers have no preferred orientation, and therefore, spin with the HARV's rotation. The theoretical spin of a non-polar carrier over one revolution of the HARV is shown in time-lapse (B). The arrow on the carrier indicates its orientation at each phase of the rotation. In the polar carrier, the forces of gravity (Fg) and buoyancy (Fb) are also nearly balanced. However, due to the presence of a buoyant pole on the carrier, they do not coincide, giving the carrier a preferred orientation (C). These polar carriers resist rotation and therefore resist spinning as the HARV turns. The theoretical stable orientation of a polar carrier over one revolution of the HARV is shown in (D). As the HARV rotates, non-polar carriers are subjected to vector-averaged gravity while polar carriers remain in normal gravity conditions. **E**: Cross-sectional diagram of the system used to encapsulate cells into non-polar and polar alginate carriers. The alginate-cell suspension (1% alginate, 2×10^6 cells/ml) was extruded by a syringe pump (100 ml/h) through an 18-gauge needle. Droplets released from the needle tip polymerized in the $CaCl_2$/NaCl bath forming carriers seeded with cells. The gelling bath was stirred to prevent the carriers from deforming upon entry. This process was used to form non-polar carriers of uniform size and sphericity. To form polarized carriers, aerosolized hollow glass particles were blown across the drop path. The glass particles were incorporated into the surface of the carriers upon polymerization.

encapsulating the cells inside spherical alginate carriers. The presence of competing sodium cations was used to reduce radial inhomogeneity in the polymerization of the carriers [Skjak-Braek et al., 1989]. A large volume (400 ml) gelling bath was used to limit changes in ion concentrations as carriers were formed. The sphericity of the carriers was maintained by reducing the surface tension of the gelling bath. This was achieved by stirring the gelling solution and positioning the entry point of the droplet midway between the vortex and the outer edge of the bath. This method was used to produce non-polar carriers. Polar carriers were generated by blowing hollow glass particles, as an aerosol, across the path of the droplets. The glass particles embedded on one side of the carriers created a buoyant pole. The carriers were removed from the cross-linking bath after 15 min and rinsed three times in 150 mM NaCl. Polar and non-polar carriers were created together in a single batch. The carriers were then incubated overnight (12–18 h) in DMEM supplemented with 10% fetal calf serum in 5% CO_2 –95% air at 37°C and used the following day.

Analysis of the Trajectory and Spin of Alginate Carriers in the HARV

The spin and trajectory of the carriers was measured by direct observation in the HARV using a high magnification video camera (Canon, Inc., Lake Success, NY). To analyze the spin and trajectories of individual carriers, the camera was mounted in an assembly that rotated in unison with the HARV, thereby allowing it to track the orientation of individual carriers. The video was then uploaded to a computer and the orientation of the carriers was measured by frame analysis. To assess the influence of carriers on each other, the experiment was repeated with large numbers of carriers. The trajectories were measured in the same way.

Culture of Osteoblast-Like Cells in Vector-Averaged Gravity

Approximately 25 polar and 25 non-polar carriers containing cells were cultured together in a single HARV (USA Synthecon, Inc., Houston, TX). All carriers were made positively buoyant by increasing the density of the culture medium with Ficoll 400 (Sigma–Aldrich Corp.,

St. Louis, MO). Ficoll was dialyzed prior to use (300 kd molecular weight cut-off for 4 days), filter sterilized, and added to the medium to produce a final concentration of 4% (w/v). In a preliminary experiment, confocal microscopy demonstrated that fluorescently labeled Ficoll was mostly excluded from the interior of the alginate carriers (data not shown). Furthermore, to ensure that this agent did not influence osteoblast viability, biocompatibility was verified biochemically (data not shown). The HARV was loaded with the carriers and filled with media (DMEM, 10% fetal calf serum, 4% Ficoll) and air bubbles were removed. The vessel was then rotated at 15 rpm for 24 h at 37°C in 5% CO_2. A rotational speed of 15 rpm was chosen as previous studies have shown measurable changes in cell function at equal or lower speeds [Kunisada et al., 1997; Sato et al., 1999; Rucci et al., 2002; Ontiveros and McCabe, 2003; Nakamura et al., 2003b; Bucaro et al., 2004]. The use of higher rotational speeds was avoided due to generation of sizeable centrifugal forces. In each experiment, the carriers were monitored visually to ensure that polar carriers did not spin about their own axes while undergoing rotation in the HARV. At the end of this period, the carriers were sorted into polar and non-polar populations and the resident cells analyzed.

Measurement of Osteoblast Gene Expression by RT-PCR

MC3T3-E1 osteoblast-like cells were isolated from carriers by solubilization of the alginate in a solution of 25 mM sodium citrate, 10 mM EDTA, 115 mM NaCl, pH 7.4 at 37°C, for 15 min. Osteoblasts were collected by centrifugation. RNA was isolated from the cell pellet and purified using QIA shredder and RNAEasy protect kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. RT-PCR was performed using Ready-To-Go RT-PCR beads (Amersham Biosciences Corp., Piscataway, NJ), which included pre-portioned buffer, nucleotides, and Taq polymerase. Temperature cycling was as follows: cDNA synthesis 30 min at 42°C, initial melt 4 min at 94°C, amplification cycle (1 min at 94°C, 1 min at 55°C, 2 min at 72°C), 10 min 72°C, >2 min at 4°C. Genes analyzed include RUNX2 (f:GCGTATTT CAGATGATGACA, r:TACCATTGGGAACTG-ATAGG), osteopontin (f: TCCTCTGAAGAAA-AGGATGA, r:CTTCATGTGAGAGGTGAGGT), osteocalcin (f:CTCTCTGACCTCACAGATGC, r:TGCTGTGACATCCATACTTG), collagen 1a (f: TTGATCTGTATCTGCCACAA,:GCTGATT-TTTCATCATAGCC). Bcl2 (f:GACCCGTGTC-TATGGATTTA, r:TGAAATATCAACCACAG-CAA) Bax (f:GAGATGAACTGGACAGCAAT, r:ATCTTCTTCCAGATGGTGAG) Alkaline phosphase f:GCTTTAAACCCAGACACAAG, r:GC-AGTAACCACAGTCAAGGT). Both GAPDH (f: CTCACTCAAGATTGTCAGCA, r: GTCATCA-TACTTGGCAGGTT), and the QuantumRNA™ universal 18S internal standard (Ambion, Inc., Austin, TX) were amplified independently as controls.

Measurement of Cell Viability

The viability of the three types of cells after culture in the HARV was determined by measuring the conversion of a tetrazolium salt (MTT) (Sigma–Aldrich) to formazan. Carriers were loaded with C3H10T1/2 pluripotential cells, MC3T3-E1 osteoblast-like cells, and MLO-Y4 and MLO-A5, osteocyte-like cells. Cells in the carriers were incubated with 0.5 mg/ml MTT in serum-free DMEM for 3 h at 37°C/5% CO_2. The carriers were rinsed in NaCl, and polar and non-polar carriers were sorted under a dissecting scope and placed into separate 1.5 ml eppendorf tubes. To dissolve the alginate, the carriers were treated with 55 mM sodium citrate, 100 mM NaCl for 10 min. The cells were then pelleted by centrifugation and resuspended in 200 μl DMSO. MTT activity was measured using a plate reader (Tecan Group Ltd., Maennedorf, Switzerland). To evaluate the effect of microgravity on the induction of apoptosis by known apoptogens following culture in the HARV for 1 day, the carriers were harvested and then treated with staurosporine (0.1–1.0 μM) or sodium nitroprusside (0.05–0.50 μM) in serum-free medium for 12 h in a static culture. Following this apoptogen treatment, MTT activity was recorded as described above to determine if cell viability was affected. Controls for this study included cells treated with serum-free medium. DNA content was measured spectrofluorometrically from cell lysates using PicoGreen (Molecular Probes, Inc., Eugene, OR). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison procedure.

Quantification of Live/Dead Cells

To determine the percentage of live and dead cells within the alginate carriers, MC3T3-E1 osteoblast-like cells were evaluated by confocal microscopy using fluorescent probes. 5-chloromethylfluorescein diacetate (CellTracker Green) was used at 10 μM and propidium iodide was used at 10 μg/ml. CellTracker Green was used to identify viable cells and propidium iodide was used to label dead or dying cells. After incubation with the dyes for 5 min, the cells were imaged with an Olympus Fluoview confocal microscope. Optical image stacks were taken at 5 μm steps to a depth of 100 μm, and rendered as an overlay.

RESULTS

Characterization of Encapsulated Osteoblasts

The electrostatic bead generator produced spherical carriers with average diameter of approximately 2 mm. There was no obvious difference in the size or the sphericity of the polar and non-polar carriers. Figure 2A shows a dark-field image of both a non-polar and a polar carrier. MC3T3-E1 cells are dispersed homogeneously within both carriers and the hollow glass particles can be seen on the surface of the polar carrier. Carriers were sorted into polar and non-polar by the presence of glass particles for evaluation of cell number and viability. The number of cells per carrier and cell viability were measured by assessing the DNA content (Fig. 2B) and the MTT activity (Fig. 2C) of cells from individual carriers. These assays revealed that there was no statistical difference either of these measurements for polar and non-polar carriers at the time of formation. To evaluate the long-term effects of culture prior to performing microgravity studies, cells in polar and non-polar carriers were maintained in a single culture dish for 5 days in static conditions, and then incubated with 5-chloromethylfluorescein

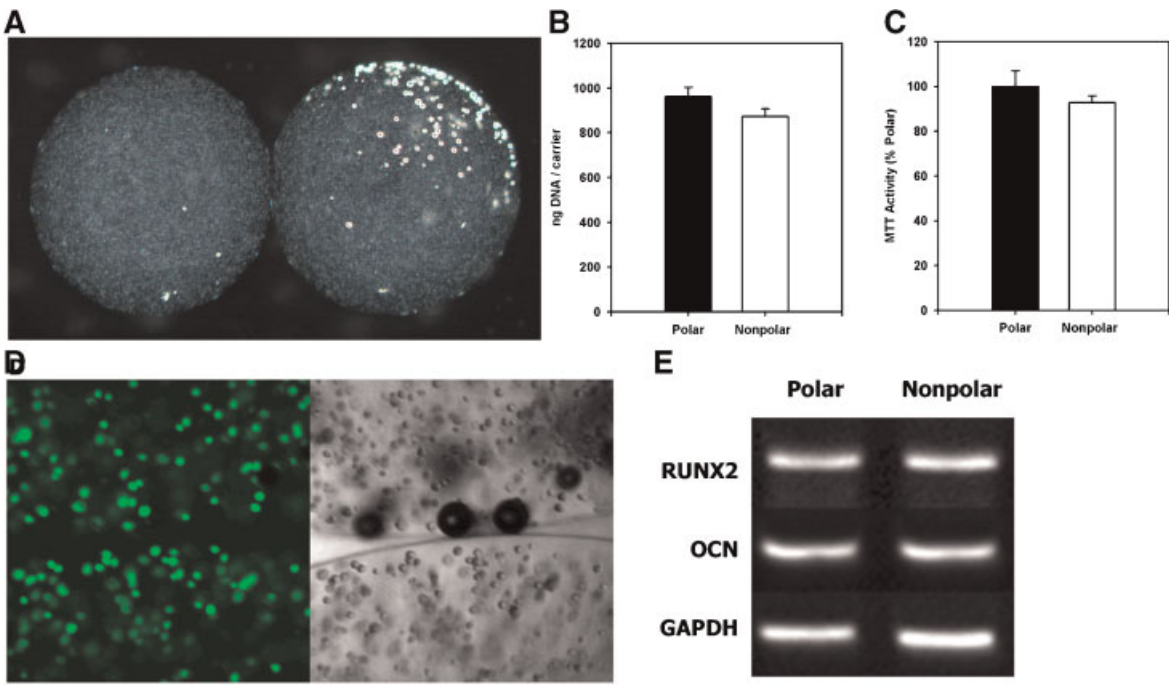

Fig. 2. Uniformity of carriers and maintenance of MC3T3-E1 viability, thiol status, and osteoblast-specific gene expression. **A**: Photograph of a non-polar (**left**) and a polar carrier (**right**) containing MC3T3-E1 cells. Note the similarity in diameter, sphericity, and cell distribution. The upper pole of the polar carrier (right) is coated with buoyant glass particles (white). **B–C**: Quantification of cell number per carrier. (B) DNA content of individual carriers was measured using the PicoGreen assay ($n = 11$). (C) Cell number was also evaluated by determination of the MTT activity of cells within the carriers ($n = 6$). Standard error is shown by error bars. The figure shows that a consistent number of cells were encapsulated into each carrier. Note that there was no significant difference in cell number between polar and non-polar carriers ($P < 0.05$). **D**: Thiol status of the cells in long-term culture. After encapsulation, cells were cultured for 5 days in static culture and incubated with CellTracker to evaluate thiol status. The extended focus confocal image stack (100 μm depth) shows that in both polar and non-polar carriers, the majority of the cells were positive for CellTracker **(left)**. Brightfield image (**right**) shows a polar carrier (**upper**) with glass particles and a non-polar carrier (**lower**). **E**: Osteoblast gene expression in alginate encapsulated osteoblasts. The expression of RUNX2 and osteocalcin was assessed by RT-PCR after 5 days. The expression of both RUNX2 and osteocalcin was maintained in cells independent of carrier type.

diacetate (CellTracker Green). The fluorescent product is retained within viable cells and provides information on the thiol redox status. Confocal image stacks (100 μm depth) of polar and non-polar carriers were generated and juxtaposed with the reflected light image (Fig. 2D). The majority of cells in both polar and non-polar carriers were positive for CellTracker, indicating that the cells preserved their thiol redox and survived in the alginate matrix. RT-PCR of the osteoblast-specific gene expression of MC3T3-E1 cells cultured in alginate carriers for 5 days demonstrated that the cells maintain osteoblast-specific gene expression (Fig. 2E). RUNX2 and osteocalcin were robustly expressed in both polar and non-polar carriers, relative to GAPDH.

Trajectory and Spin of Carriers

The trajectory of the carriers within the HARV was recorded using a video camera mounted in front of a spinning HARV and the location tracked by video analysis. The measured trajectories demonstrate that the carriers quickly stabilize in positions that prevent their collision with the walls of the HARV (Fig. 3). These empirical findings correlate generally well with the results of a numerical simulation of a single carrier in the HARV.

The spin of the carriers was analyzed using high magnification video recorded with a camera that moved in unison with the HARV's rotation. Spin was measured by tracking the position of a neutrally buoyant opaque particle inside each carrier. Since the camera rotated with the HARV, it was necessary to subtract the camera's rotation to display the instantaneous orientation of the carriers as seen by a laboratory observer. The series of frames shown capture each carrier's spin about its own axis during one revolution of the HARV (Fig. 4). The frames depicting the behavior of a polar carrier show that as the HARV rotates, the orientation of the carrier remains unchanged (Fig. 4A). The buoyant pole can be seen always at the top end of the carrier. Frames depicting the behavior of a non-polar carrier show that the carrier undergoes a counter-clockwise rotation, spinning essentially at the same rate as the HARV (Fig. 4B). The sine of the angle of rotation of the polar and non-polar carriers is shown without correction for the rotation of the camera (Fig. 4C). Since the data is in the rotating frame of reference (not corrected for the camera/HARV rotation), the plot for the polar carrier mimics the rotation of the HARV. The plot for the non-polar carrier shows that, although there is a randomized but slight departure from absolute vertical stability, there is dynamic stabilization in the rotating frame of reference. The same data was corrected for the rotation of the camera to display the rotations of a polar carrier and a non-polar carrier in the inertial frame of reference (Fig. 4D). The non-polar carrier spins at nearly the same rate as the HARV while the polar carrier exhibits little variation in its orientation. The data demonstrate that polar carriers maintain a stable orientation, while non-polar carriers spin on their horizontal axis in near unison with the HARV's rotation. Therefore, over each revolution of the HARV, the time-averaged gravity vector is approximately unit gravity for the polar carrier and close to zero for the non-polar carrier. These results were consistently observed in several independent studies performed with HARV speeds ranging from 3 to 15 rpm.

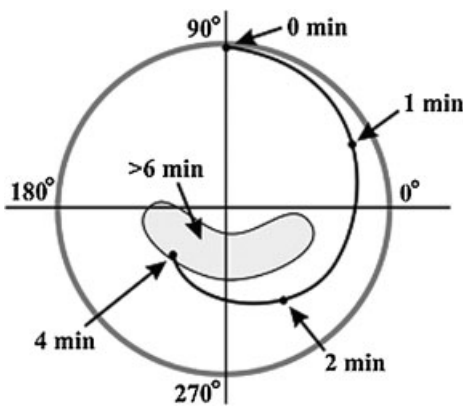

Fig. 3. Analysis of the trajectory of the carriers in the HARV. The actual trajectories of alginate carriers were recorded using a camera that rotated in unison with the HARV. A representative trajectory is plotted in the rotating frame of reference. The actual trajectory closely followed the path predicted by direct numerical simulation, never colliding with the wall of the HARV. After 6 min, the carrier reached a stable region in the HARV, from which it did not deviate (gray). The carrier shown was tracked for 48 h. This study was repeated with polar and non-polar carriers. All carriers followed a similar trajectory.

Effect of Vector-Averaged Gravity on Osteoblast Viability and Gene Expression

To determine if vector-averaged gravity modulated gene expression, MC3T3-E1 cells were cultured in the HARV for 5 days and then subjected to RT-PCR analysis. No differences were observed for the expression of RUNX2,

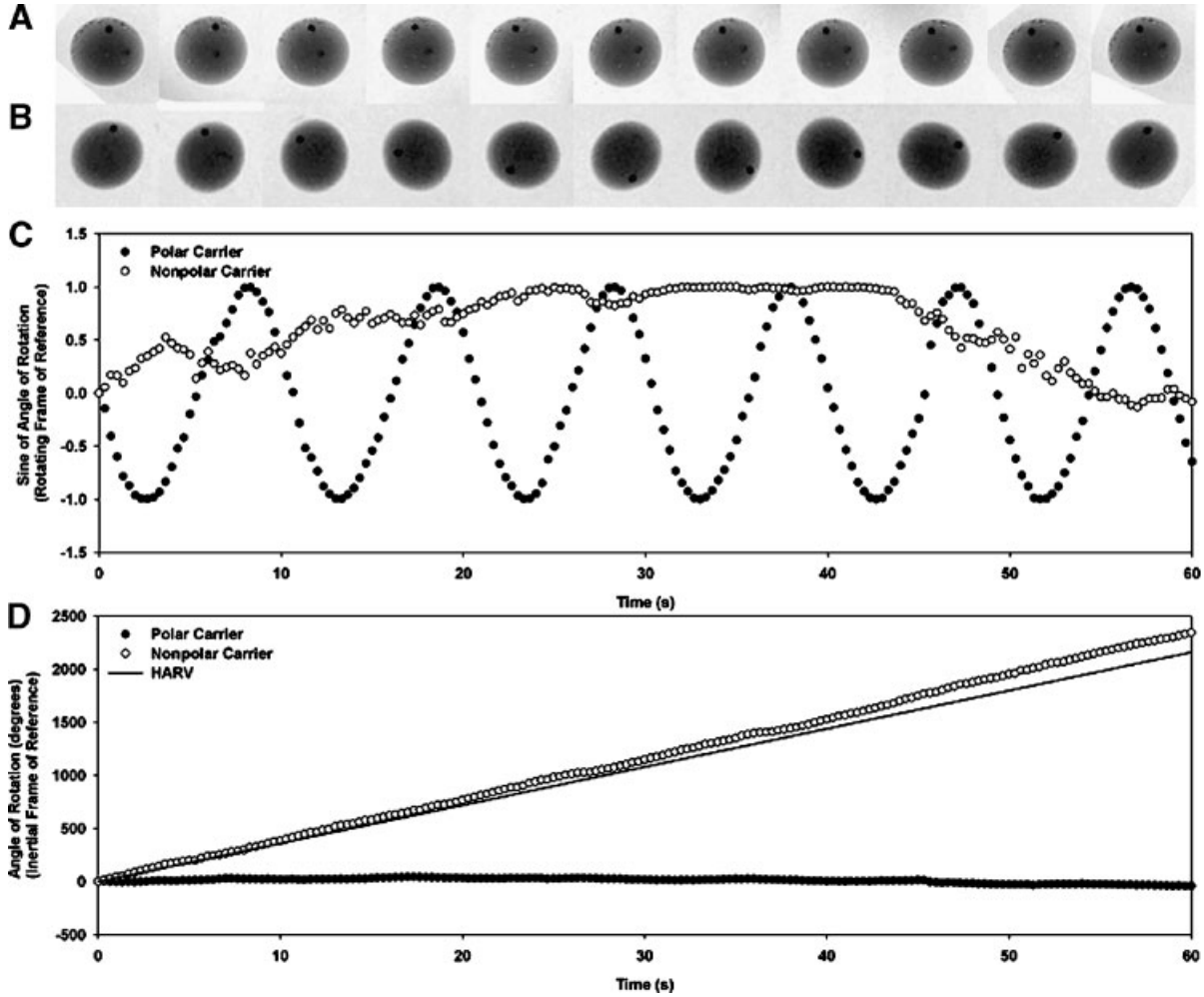

Fig. 4. Spin of polar and non-polar carriers in the HARV. A video camera was mounted to the front face of the HARV to monitor individual carriers. Frame by frame analysis was performed to determine the spin of the carriers. Blue dextran was used to aid in visualizing the carriers and orientation was measured by monitoring the position of an opaque particle in the carrier. Frames from a single revolution of the HARV of a polar carrier (**A**) and a non-polar carrier (**B**) were corrected for the rotation of the camera. The polar carrier does not spin and therefore is in normal gravity conditions. The non-polar carrier rotates on its horizontal axis at a constant angular velocity at the same rate as the HARV. **C**: The sine of the angle of rotation of a polar carrier and a non-polar carrier that have not been corrected for the rotation of the camera is plotted versus time. The plot for the polar carrier in the rotating frame of reference mimics the rotation of the HARV. The plot for the non-polar carrier in the rotating frame of reference shows that it is synchronized with the HARV's rotation, but is characterized by some small, unpredictable spin. **D**: The angle of rotation, corrected for the rotation of the HARV is plotted versus time. Note that the non-polar carrier spins at the same rate as the HARV rotates. The polar carrier does not spin with the HARV.

osteopontin, osteocalcin, or collagen 1 relative to the level of 18S ribosomal subunit (Fig. 5). Furthermore, we detected no changes in the expression of the pro-apoptotic gene, Bax, or the anti-apoptotic gene, Bcl-2. A decrease in the expression of alkaline phosphatase was detected in cells cultured in non-polar carriers relative to polar carriers. This is consistent with observations made following spaceflight studies [Collet et al., 1997; Caillot-Augusseau et al., 1998]. Control experiments demonstrated that cells maintained in static culture in polar and non-polar carriers did not show any evidence of differences in expression of these genes (data not shown). To determine if vector-averaged gravity induced cell death, the cells were monitored for viability using the MTT assay. Polar and non-polar carriers, both in the HARV and in static culture, did not show any evidence of a difference in MTT activity at both day 1 and day 5 (Fig. 6A,B). To determine if longer exposure to vector-averaged gravity affects cell viability, the culture time was extended to 2 weeks and the fetal calf serum

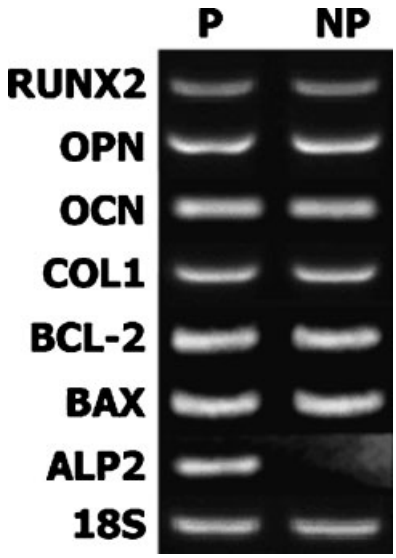

Fig. 5. Expression of osteoblast-specific genes in vector-averaged and normal gravity. Cells in polar and non-polar carriers were cultured in the HARV for 5 days, sorted, and subjected to RT-PCR. Cells from both carrier types were analyzed for the expression of RUNX2, osteopontin (OPN), osteocalcin (OCN), collagen 1a (COL1), Bcl2, Bax, and alkaline phosphatase (ALP2). The same expression levels for RUNX2, OPN, OCN, COL1, Bcl-2, and Bax relative to 18S, were observed in cells from both carrier types. The expression of alkaline phosphatase was substantially lower in non-polar carriers relative to polar carriers, indicating that its expression was suppressed by vector-averaged gravity.

concentration was reduced to 1%. At the end of 2 weeks, no significant differences in MTT activity were observed between polar and non-polar carriers (Fig. 6C). Moreover, when stained with CellTracker Green and propidium iodide, no differences were observed in the number of propidium iodide or CellTracker positive cells in the polar and non-polar carriers (Fig. 6D).

While the results of the studies described above indicate that vector-averaged gravity does not directly induce osteoblast cell death, the possibility remained that it could exacerbate their sensitivity to apoptogens. To assess sensitivity, osteoblasts representative of different stages of the differentiation pathway were each cultured in the HARV in polar and non-polar carriers for 1 day, and then treated with low levels of the known apoptogens, staurosporine (0.1–1.0 μM) or sodium nitroprusside (0.05–0.50 μM), in serum-free medium overnight. When maintained in the polar and non-polar carriers, no differences in the in percentage of dead cells were detected (Fig. 7A,B). Accordingly, exposure to both staurosporine and sodium nitroprusside produced the same dose-dependent increase in cell death. The osteocyte-like MLO-A5 cells (provided by Dr. Linda Bonewald), (Fig. 7C,D) and the osteoblast progenitor-like C3H10T-1/2 cells, (Fig. 7E,F) which maintain the ability to commit to a number of pathways also showed no significant differences in cell death when maintained in polar or non-polar carriers. Another osteocyte-like cell line, MLO-Y4 cells (provided by Dr. Linda Bonewald), produced similar results to those presented for MLO-A5 cells (data not shown). These results clearly indicate that vector-averaged gravity does not sensitize cells at different stages of the osteoblast lineage to the activation of programmed cell death.

DISCUSSION

The primary objective of this investigation was to address the hypothesis that microgravity directly induces osteoblast apoptosis, using clinorotation (vector-averaged gravity) to simulate aspects of microgravity. Cognizant of the drawbacks associated with the use of conventional clinostats, we designed a novel system in which osteoblasts were embedded in alginate carriers. By using rotationally stabilized carriers, we were able to control for extraneous factors such as fluid flow-induced shear stress, vibration, and convective mixing in the HARV. Using video microscopy, we confirmed the clinorotation of the carriers and ascertained their trajectories were free from collisions with the wall of the HARV. In contrast to previous studies, we found that vector-averaged gravity, even for extended time periods, did not reduce osteoblast viability (MC3T3-E1 cells). Furthermore, neither osteoblasts, nor progenitor-like cells, nor osteocyte-like cells were sensitized to apoptogens by exposure to vector-averaged gravity. We observed some differences in the phenotypic expression of these cells; specifically, RT-PCR analysis indicated that there was a decrease in alkaline phosphatase expression in simulated microgravity. These findings suggest that vector-averaged gravity alone is insufficient to induce or modulate the susceptibility of osteoblasts to agents that induce apoptosis.

The new carrier culture system was designed to minimize confounding factors that might exacerbate cell death. Alginate encapsulation of the cells served several critical functions. First,

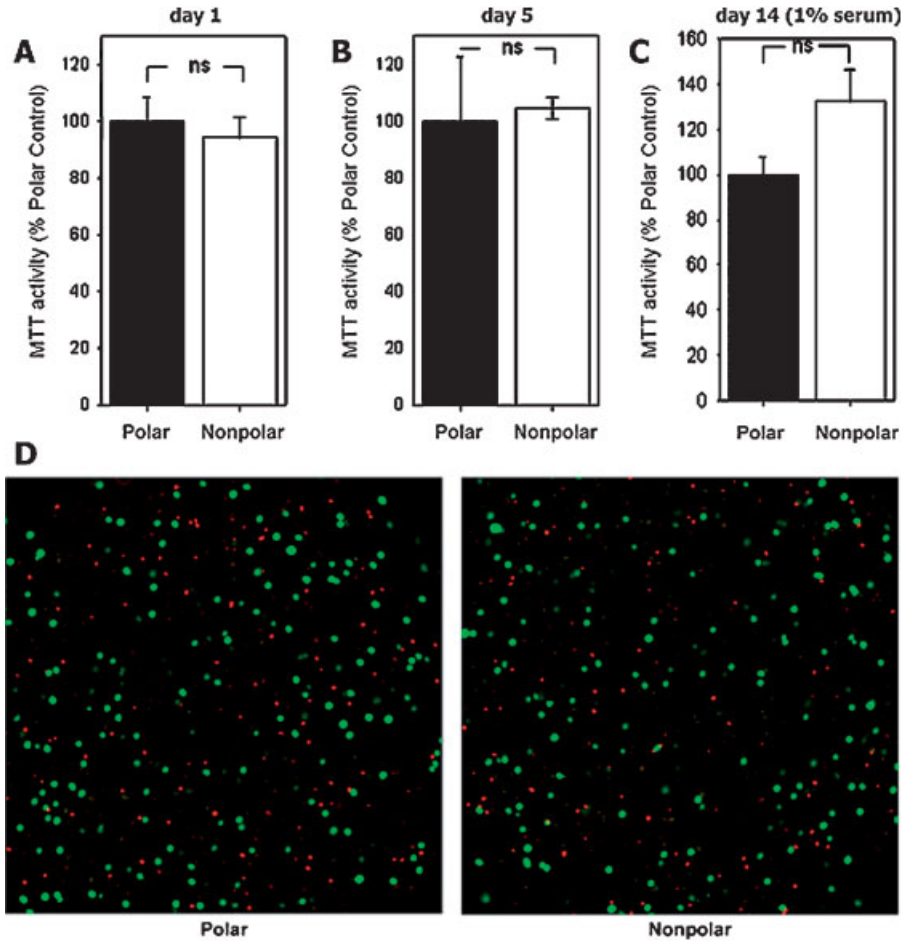

Fig. 6. Viability of MC3T3-E1 cells in vector-averaged gravity. **A**, **B**: Cells in polar and non-polar carriers were cultured in the HARV in 10% serum for 1 day (A) or 5 days (B). The MTT activity of the cells in polar and non-polar was measured at the end of the culture period. In both experiments, no significant differences in viability were observed between polar and non-polar carriers, indicating that vector-averaged gravity did not induce cell death. **C**, **D**: Cells in polar and non-polar carriers were cultured in 1% serum for 2 weeks in vector-averaged gravity and normal gravity and viability was determined by the MTT assay (C) and CellTracker staining (D). No significant difference was noted in the MTT activity of the polar and non-polar cells. [CellTracker (green) fluorescence indicates viable cells and propidium iodide (red) fluorescence indicates dead or dying cells with permeable membranes.] No difference in CellTracker positive and propidium iodide positive fluorescence was observed between cells in polar (**left**) and non-polar carriers (**right**) (Bars indicate means and standard errors).

since the cells were inside of the carriers, they were protected from the effects of collisions with other carriers and with the walls of the HARV. Second, cells were shielded from the fluid flow present on the surface of the carriers while receiving sufficient nutrient supply, due to the low permeability and high diffusivity of alginate beads, respectively [Amsden and Turner, 1999]. Third, alginate encapsulation provided a three-dimensional scaffold that supported the differentiated phenotype [Majmudar et al., 1991]. Inside the carriers, cells maintained normal metabolic activity and expressed key osteoblast-specific genes, as evidenced by biochemical assessments and RT-PCR. Finally, of particular significance to the studies reported herein, dying cells were not lost during the culture period since all cells were confined within the alginate carriers.

The most significant advancement in our system was the use of rotationally stabilized, polarized carriers to culture cells in normal gravity conditions in the HARV. This approach ensured that the effects observed were due to vector-averaged gravity, and were not confounded by other factors. The problem of adequate controls for extraneous stimuli has been addressed by others. For example, to control for shear forces and vibration, Sarkar et al. [2000a,b] cultured control (normal gravity) cells in a clinostat with a vertical axis of rotation. While this approach has advantages

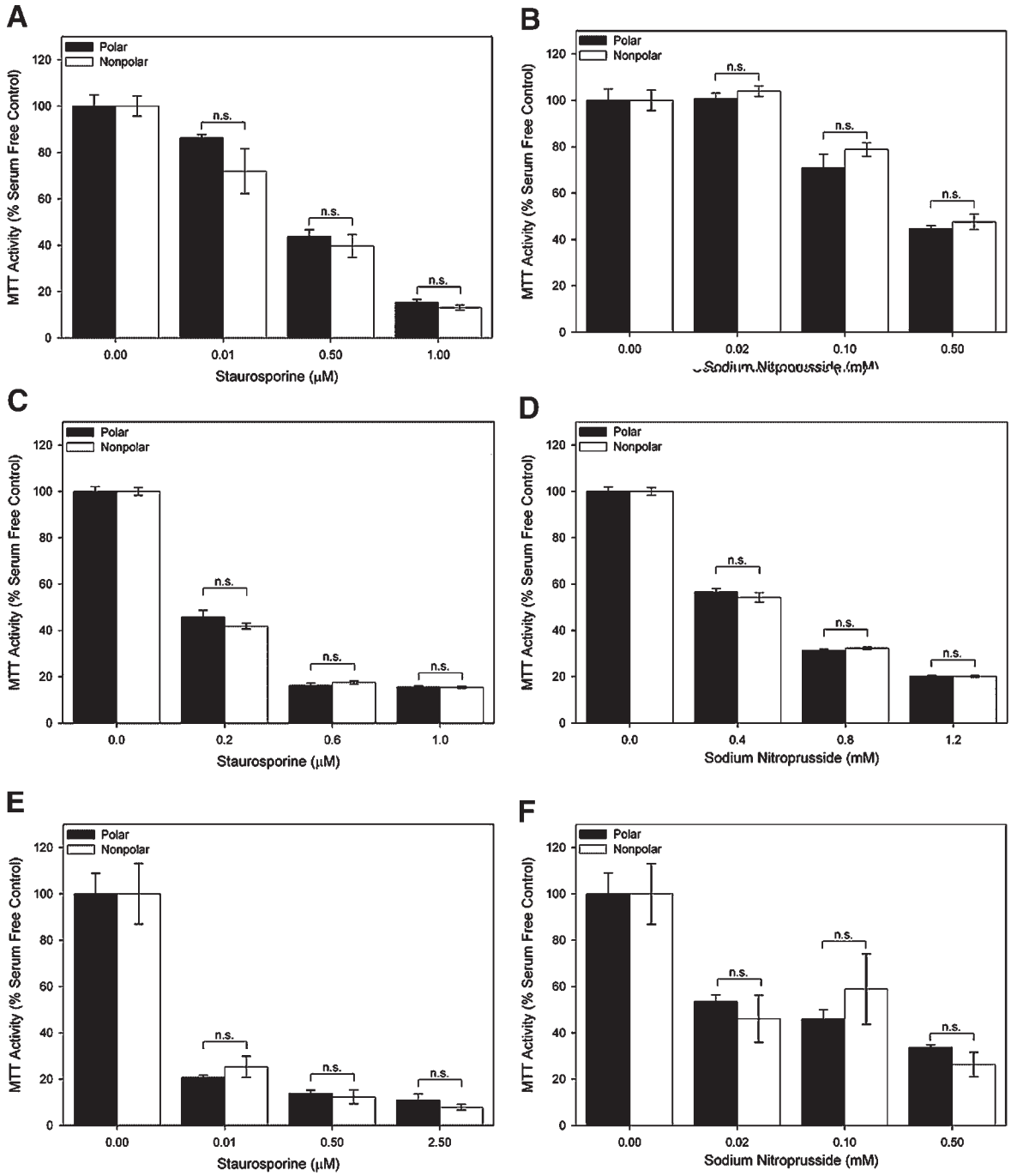

Fig. 7. Sensitivity of osteblast-like cells (MC3T3-E1) (**A–B**), osteocyte-like cells (MLO-A5) (**C–D**), and C3H10T1/2 pluripotent stem cells (**E–F**) to apoptogens after culture in vector-averaged gravity. After 24 h culture in the HARV, cells were challenged overnight with staurosporine (A, C, E) and sodium nitroprusside (B, D, F) and viability was determined by the MTT procedure. For each apoptogen, a dose dependent reduction in MTT activity was observed. However, no significant differences were seen between polar and non-polar carriers at any of the doses for any cell type. MLO-Y4 cells showed similar results to the data presented here for MLO-A5 cells. Results are normalized to serum-free controls (Bars indicate means and standard errors).

over static controls, there are a number of shortcomings. For example, it is not clear if fluid flow-induced shear forces could play a role in the detachment of cells from the substrate. Additionally, the fluid dynamics may generate differential shear forces in the vertical and horizontal configurations due to asymmetries, such as air bubbles. The system described herein, using encapsulation of the cells to minimize shear forces and the use of polar

carriers to maintain osteoblasts in normal gravity, provides a more controlled approach to evaluate the effects of vector-averaged gravity. Furthermore, use of a single HARV to co-culture the cells in vector-averaged gravity and normal gravity simultaneously, eliminates many of the variables that characterized earlier experimental systems. It should be noted that the morphology of the osteoblasts in alginate is distinctly different from that on flat substrates which may influence the effects of vector-averaged gravity.

The influence of vector-averaged gravity on cell survival was evaluated using three assays. First, following culture in vector-averaged gravity, the MTT assay was used to assess cell viability. The viability of osteoblasts cultured in vector-averaged gravity was the same as cells maintained in normal gravity, even during extended cultures of 14 days in reduced serum. Second, following culture, the cells were stained with CellTracker (5-chloromethylfluorescein diacetate), an indicator of intracellular glutathione S-transferase activity of the cultured cells. No difference was observed in the activity of this enzyme and, by inference, the thiol redox status of the cells. Finally, a challenge with two apoptogens failed to show any difference in the sensitivity of cells maintained in normal or vector-averaged gravity environments to the induction of cell death. Therefore, vector-averaged gravity does not cause osteoblast cell death, or change their sensitivity to well characterized apoptogens. As we utilized cells at three differentiation stages, we can state that modulation of sensitivity to apoptogens by gravitational conditions is not maturation-dependent. None of these studies provided any support for the hypothesis that vector-averaged gravity affects osteoblast survival.

The results of the biochemical studies are appreciably different from those described by other workers. For example, Rucci et al. [2002] reported that there were elevated levels of alkaline phosphatase expression and activity, and raised expression of osteopontin, osteocalcin, and BMP-4 in RWV studies. Furthermore, nucleosome-size fragmentation was observed, although p53, bcl-2/bax, and caspase 8 pathways were not activated [Rucci et al., 2002]. Nakamura et al. reported, using human osteoblastic cells that expression of differentiation markers and the ratios of Bax/Bcl-2 mRNA levels were significantly increased in vector-averaged gravity cells, relative to cells cultured in static conditions. However, no changes in proliferation or apoptosis were observed [Nakamura et al., 2003a]. It should be noted that all of the above observations were made relative to static cultures, and therefore, did not fully account for extraneous factors present in the microgravity cultures. In another study, Sarkar et al. [2000b] noted that 35% of ROS 17/2.8 cells under clinorotation (50 rpm) had detached from the surface or underwent apoptotic death after 24 h.

The importance of adequate control is also evident from our earlier study on osteoblasts cultured in vector-averaged gravity [Bucaro et al., 2004]. In that study, the cells were embedded in alginate carriers and cultured in the HARV. However, normal gravity control cells were maintained in static culture, and therefore, we were unable to control for the other extraneous factors present in the HARV that may serve to exacerbate cell death. In these experiments, although osteoblasts remained viable after culture in vector-averaged gravity, we noted an increase in sensitivity to apoptogen treatment relative to static controls. We also observed a generalized reduction in osteoblast-specific gene expression. In contrast, the experiments described in the present study, which accounted for variables introduced by culture in the HARV, demonstrated no effects on viability or the expression of bone-specific genes, with the exception of alkaline phosphatase. Thus, we conclude that in a well-controlled system, over a long culture period, vector-averaged gravity (15 rpm) does not induce cell death, influence the metabolic state of the cells or generally inhibit expression of the mature osteoblast phenotype.

A mechanistic understanding of bone loss during spaceflight is needed for the development of countermeasures and may provide insights into the pathologies of other osteopenic states. Although skeletal unloading undoubtedly provokes bone loss in space [Carmeliet et al., 2001; Bikle et al., 2003; Yuge et al., 2003], in-flight countermeasures aimed at restoring normal skeletal loads through exercise have shown limited success in preventing osteopenia [Rambaut and Johnston, 1979; Convertino, 1996]. This suggests that in addition to the effect of unloading on bone, there may be direct effects of microgravity on the cells of bone. Space flown studies on isolated osteoblasts have

shown a broad array of responses of these cells to microgravity, including growth inactivation and changes in phenotypic expression [Guignandon et al., 1995; Hughes-Fulford and Lewis, 1996; Carmeliet et al., 1997, 1998; Guignandon et al., 1997; Hughes-Fulford et al., 1998; Hughes-Fulford and Gilbertson, 1999; Kumei et al., 1999; Harris et al., 2000; Landis et al., 2000]. In addition, histological examination of space-flown animals indicates a reduction in the number of osteoblasts found in bone [Jee et al., 1983; Wronski et al., 1987; Turner et al., 1995]. Several markers of the osteoblastic phenotype were unmodified by simulated microgravity in our system. Furthermore, our own results suggest that, in vitro, osteoblast cell death is not directly modulated by microgravity.

If osteoblast differentiation state, redox properties, or sensitivity to apoptogens is unaffected by microgravity, then the question remains: why is there a loss of bone mass during spaceflight? It should be noted that vector-averaged gravity does not mimic all aspects of microgravity, such as the lack of thermal convection and reduced hydrostatic pressure. Furthermore, in vivo, bone cells exist in a specialized microenvironment that is not fully recapitulated in the cell culture experiments. It is plausible that osteoblast viability may be indirectly affected by microgravity. Our own previous work has demonstrated that components of the extracellular matrix of bone, mineral ions [Adams et al., 2001] and RGD containing peptides [Perlot et al., 2002], serve as physiologic mediators of osteoblastic apoptosis. Furthermore, other work that is ongoing in our lab has identified significant effects of simulated microgravity on the function of osteoclasts in organ culture. Taken together, microgravity may increase osteoclast mediated resorption of bone, which in turn, could increase apoptosis of osteoblasts as matrix apoptogens are released. Therefore, further examination of the effects of microgravity on bone cells may require investigations of the interplay between osteocytes, osteoblasts and osteoclasts using more complex cell culture systems, organ culture studies, and whole animal models.

ACKNOWLEDGMENTS

This work was supported by NASA grant: NAG9-1400, NIH grants: DE-13319, DE-10875, and DE-05748, and the DOD grant: DAMD17-03-1-0713. MLO-A5 and Y4 cells kindly provided by Dr. Linda Bonewald.

REFERENCES

Adams CS, Mansfield K, Perlot RL, Shapiro IM. 2001. Matrix regulation of skeletal cell apoptosis—Role of calcium and phosphate ions. J Biol Chem 276:20316–20322.

Amsden B, Turner N. 1999. Diffusion characteristics of calcium alginate gels. Biotechnol Bioeng 65:605–610.

Atkov OY. 1992. Some medical aspects of an 8-month's space flight. Adv Space Res 12:343–345.

Bikle DD, Sakata T, Halloran BP. 2003. The impact of skeletal unloading on bone formation. Gravit Space Biol Bullet 16:45–54.

Bucaro MA, Fertala J, Adams CS, Steinbeck M, Ayyaswamy P, Mukundakrishnan K, Shapiro IM, Risbud MV. 2004. Bone cell survival in microgravity: Evidence that modeled microgravity increases osteoblast sensitivity to apoptogens. Ann N Y Acad Scii 1027:64–73.

Caillot-Augusseau A, Lafage-Proust MH, Soler C, Pernod J, Dubois F, Alexandre C. 1998. Bone formation and resorption biological markers in cosmonauts during and after a 180-day space flight (Euromir 95). Clin Chem 44: 578–585.

Carmeliet G, Nys G, Bouillon R. 1997. Microgravity reduces the differentiation of human osteoblastic MG-63 cells. J Bone Mineral Res 12:786–794.

Carmeliet G, Nys G, Stockmans I, Bouillon R. 1998. Gene expression related to the differentiation of osteoblastic cells is altered by microgravity. Bone 22 (Suppl 5):139S–143S.

Carmeliet G, Vico L, Bouillon R. 2001. Space flight: A challenge for normal bone homeostasis. Crit Rev Eukaryotic Gene Expr 11:131–144.

Collet P, Uebelhart D, Vico L, Moro L, Hartmann D, Roth M, Alexandre C. 1997. Effects of 1- and 6-month spaceflight on bone mass and biochemistry in two humans. Bone 20:547–551.

Convertino VA. 1996. Exercise as a countermeasure for physiological adaptation to prolonged spaceflight. Med Sci Sports Exercise 28:999–1014.

Dai Z, Li Y, Ding B, Zhang Y, Liu W, Liu P. 2004. Effects of clinorotation on COL1A1—EGFP gene expression. Sci China Ser C, Life Sci 47:203–210.

Granet C, Boutahar N, Vico L, Alexandre C, Lafage-Proust MH. 2001. MAPK and SRC-kinases control EGR-1 and NF-kappa B inductions by changes in mechanical environment in osteoblasts. Biochem Biophys Res Commun 284:622–631.

Granet C, Vico AG, Alexandre C, Lafage-Proust MH. 2002. MAP and src kinases control the induction of AP-1 members in response to changes in mechanical environment in osteoblastic cells. Cell Signal 14:679–688.

Guignandon A, Usson Y, Laroche N, Lafage-Proust MH, Sabido O, Alexandre C, Vico L. 1997. Effects of intermittent or continuous gravitational stresses on cell-matrix adhesion: Quantitative analysis of focal contacts in osteoblastic ROS 17/2.8 cells. Exper Cell Res 236:66–75.

Guignandon A, Vico L, Alexandre C, Lafage-Proust MH. 1995. Shape changes of osteoblastic cells under gravitational variations during parabolic flight relationship with PGE2 synthesis. Cell Struct Funct 20:369–375.

Harris SA, Zhang M, Kidder LS, Evans GL, Spelsberg TC, Turner RT. 2000. Effects of orbital spaceflight on human osteoblastic cell physiology and gene expression. Bone 26: 325–331.

Hughes-Fulford M. 2002. Physiological effects of microgravity on osteoblast morphology and cell biology. Adv Space Biol Med 8:129–157.

Hughes-Fulford M, Gilbertson V. 1999. Osteoblast fibronectin mRNA, protein synthesis, and matrix are unchanged after exposure to microgravity. FASEB J 13 (Supp1): S121–S127.

Hughes-Fulford M, Lewis ML. 1996. Effects of microgravity on osteoblast growth activation. Exper Cell Res 224:103–109.

Hughes-Fulford M, Tjandrawinata R, Fitzgerald J, Gasuad K, Gilbertson V. 1998. Effects of microgravity on osteoblast growth. Gravit Space Biol Bullet: Pub Am Soc Gravit Space Biol 11:51–60.

Hughes-Fulford M, Rodenacker K, Jutting U. 2006. Reduction of anabolic signals and alteration of osteoblast nuclear morphology in microgravity. J Cell Biochem 99: 435–449.

Jee WS, Wronski TJ, Morey ER, Kimmel DB. 1983. Effects of spaceflight on trabecular bone in rats. Am J Physiol 244:R310–R314.

Klaus DM. 2001. Clinostats and bioreactors. Gravit Space Biol Bull 14:55–64.

Klokk TI, Melvik JE. 2002. Controlling the size of alginate gel beads by use of a high electrostatic potential. J Microencapsul 19:415–424.

Kumei Y, Akiyama H, Hirano M, Shimokawa H, Morita S, Mukai C, Nagaoka S. 1999. Space flight modulates signal transduction pathway of growth factor receptors in rat osteoblasts. Uchu Seibutsu Kagaku 13:142–143.

Kunisada T, Kawai A, Inoue H, Namba M. 1997. Effects of simulated microgravity on human osteoblast-like cells in culture. Acta Medica Okayama 51:135–140.

Landis WJ, Hodgens KJ, Block D, Toma CD, Gerstenfeld LC. 2000. Spaceflight effects on cultured embryonic chick bone cells. J Bone Miner Res 15:1099–1112.

Mack PB, LaChance PA, Vose GP, Vogt FB. 1967. Bone demineralization of foot and hand of gemini-titan IV, V and VII astronauts during orbital flight. Am J Roentgenol Radium Ther Nucl Med 100:503–511.

Majmudar G, Bole D, Goldstein SA, Bonadio J. 1991. Bone cell culture in a three-dimensional polymer bead stabilizes the differentiated phenotype and provides evidence that osteoblastic cells synthesize type III collagen and fibronectin. J Bone Miner Res 6:869–881.

Mukundakrishnan K, Ayyaswamy PS, Risbud M, Hu HH, Shapiro IM. 2004. Modeling of phosphate ion transfer to the surface of osteoblasts under normal gravity and simulated microgravity conditions. Ann N Y Acad Sci 1027:85–98.

Nakamura H, Kumei Y, Morita S, Shimokawa H, Ohya K, Shinomiya K. 2003a. Antagonism between apoptotic (Bax/Bcl-2) and anti-apoptotic (IAP) signals in human osteoblastic cells under vector-averaged gravity condition. Ann N Y Acad Sci 1010:143–147.

Nakamura H, Kumei Y, Morita S, Shimokawa H, Ohya K, Shinomiya K. 2003b. Suppression of osteoblastic phenotypes and modulation of pro- and anti-apoptotic features in normal human osteoblastic cells under a vector-averaged gravity condition. J Med Dental Sci 50:167–176.

Oganov VS, Grigor'ev AI, Voronin LI, Rakhmanov AS, Bakulin AV, Schneider VS, LeBlanc AD. 1992. Bone mineral density in cosmonauts after flights lasting 4. 5–6months on the Mir orbital station. Aviakosmicheskaia i Ekologicheskaia Meditsina 26:20–24.

Ontiveros C, McCabe LR. 2003. Simulated microgravity suppresses osteoblast phenotype, Runx2 levels and AP-1 transactivation. J Cell Biochem 88:427–437.

Patel MJ, Liu W, Sykes MC, Ward NE, Risin SA, Risin D, Jo H. 2007. Identification of mechanosensitive genes in osteoblasts by comparative microarray studies using the rotating wall vessel and the random positioning machine. J Cell Biochem [Epub ahead of print].

Perlot RL, Shapiro IM, Mansfield K, Adams CS. 2002. Matrix regulation of skeletal cell apoptosis II: Role of arg-gly-asp-containing peptides. J Bone Miner Res 17:66–76.

Rambaut PC, Johnston RS. 1979. Prolonged weightlessness and calcium loss in man. Acta Astronautica 6:1113–1122.

Rucci N, Migliaccio S, Zani BM, Taranta A, Teti A. 2002. Characterization of the osteoblast-like cell phenotype under microgravity conditions in the NASA-approved Rotating Wall Vessel bioreactor (RWV). J Cell Biochem 85:167–179.

Sarkar D, Nagaya T, Koga K, Kambe F, Nomura Y, Seo H. 2000a. Rotation in clinostat results in apoptosis of osteoblastic ROS 17/2.8 cells. J Gravitat Physiol: J Int Soc Gravit Physiol. 7:71–72.

Sarkar D, Nagaya T, Koga K, Nomura Y, Gruener R, Seo H. 2000b. Culture in vector-averaged gravity under clinostat rotation results in apoptosis of osteoblastic ROS 17/2.8 cells. J Bone Miner Res 15:489–498.

Sato A, Hamazaki T, Oomura T, Osada H, Kakeya M, Watanabe M, Nakamura T, Nakamura Y, Koshikawa N, Yoshizaki I, Aizawa S, Yoda S, Ogiso A, Takaoki M, Kohno Y, Tanaka H. 1999. Effects of microgravity on c-fos gene expression in osteoblast-like MC3T3-E1 cells. Adv Space Res 24:807–813.

Sievers A, Hejnowicz Z. 1992. How well does the clinostat mimic the effect of microgravity on plant cells and organs? ASGSB Bullet 5:69–75.

Skjak-Braek G, Grasdalen H, Smidrod O. 1989. Inhomgenous polysaccharide ionic gels. Carbohydrate Polym 10: 31–54.

Turner RT, Evans GL, Wakley GK. 1995. Spaceflight results in depressed cancellous bone formation in rat humeri. Aviat Space Environ Med 66:770–774.

Vose GP. 1974. Review of roentgenographic bone demineralization studies of the Gemini space flights. Am J Roentgenol Radium Ther Nucl Med 121:1–4.

Wronski TJ, Morey-Holton ER, Doty SB, Maese AC, Walsh CC. 1987. Histomorphometric analysis of rat skeleton following spaceflight. Am J Physiol 252:R252–R255.

Yuge L, Hide I, Kumagai T, Kumei Y, Takeda S, Kanno M, Sugiyama M, Kataoka K. 2003. Cell differentiation and p38(MAPK) cascade are inhibited in human osteoblasts cultured in a three-dimensional clinostat. In Vitro Cellular & Developmental Biology. Animal 39:89–97.