Experiments with osteoblasts cultured under varying orientations with respect to the gravity vector

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Abstract

Substrate attachment is crucial for normal growth and differentiation of many cell types. To better understand the role of gravity in osteoblast attachment and growth *in vitro*, 17-day-old embryonic chick calvarial osteoblasts were subjected to directional variations with respect to gravity. Osteoblasts, grown in MEM or DME supplemented with 10% FBS and attached to type I collagen-coated coverslips, were loaded into cylindrical containers completely filled with medium and oriented so that cells were either atop or beneath, or coverslips continuously rotated (~2 rpm) in a clinostat, thereby continuously changing their orientation with respect to gravity. Cells in these three conditions were collected daily for up to 6 days, and cell viability, two osteoblast functions, and proliferation were assessed. Data suggest the number and function of attached osteoblasts is unaltered by inversion or clino-rotation in initially confluent cultures. In sparsely plated cultures, however, osteoblast viability was significantly decreased (~50%) in inverted and rotated cultures during the first 3 days of sampling, but from days 4–6 no significant difference was found in viable cell number for the three conditions. Decreases in viable cell number within the first days of the experiments could result from death followed by detachment, detachment followed by death, differences in proliferation rate, or lag-phase duration. To help distinguish among these, BrdU labeling for 2 or 24 hr was used to assess cell proliferation rate. Log-phase growth rates were calculated and were unchanged among the three conditions tested. These results point to an increase in lag-phase duration in inverted and rotated cultures. In summary, changing the cell-substrate attachment direction with respect to gravity causes an immediate response in the form of diminished viable osteoblast number in sparse, early cultures, but the effect disappears after 3–4 days and does not occur in mature, confluent cultures.

Abbreviations: A – substrate attachment, APase – alkaline phosphatase, BrdU – 5-Bromo-2'-Deoxyuridine, DME – Dulbecco's Modified Eagle's Medium, FBS – Fetal Bovine Serum, FPA – Fluid Processing Apparatus, G – gravity vector, MEM – Minimal Essential Medium Eagle

Introduction

Bone loss and its associated complications have been identified by NASA and gravitational physiologists as one of the fundamental physiologic risks or road-blocks of extended duration space travel. Human bone loss during spaceflight, although variable, is signifi-

cant (Holick 1998; Miyamoto et al. 1998; Vico et al. 1998). Osteoblasts *in vivo* and *in vitro* are also altered as a result of low gravity (Carmeliet et al. 1998; Forkheim and Schenker 1998; Guignandon et al. 2001; Hughes-Fulford et al. 1998; Hughes-Fulford and Lewis 1996; Kaplansky et al. 1991; Landis et al. 2000; Roberts et al. 1981, 1987; Vico et al. 1998).

Because of the limited spaceflight experimentation opportunities, several investigators have simulated some of the aspects of microgravity by growing osteoblast-like cells in clinostats (vector-averaged gravity). Proliferation of osteoblast-like cells cultured in clinostats was depressed in two separate investigations (Zhang et al. 1998; Sato et al. 1999). One study showed that osteosarcoma ROS17/2.8 cells became apoptotic when clino-rotated (Sarkar et al. 2000). Another investigation described a decrease in differentiation specific gene expression in human osteoblastic cells cultured in clinostats (Kunisada et al. 1997). Finally, a separate study begins to elucidate an altered signal transduction pathway in clino-rotated osteosarcoma ROS17/2.8 cells (Granet et al. 2001).

In this study, a single cellular trait, the attachment of cells to their substrate, was examined in osteoblasts subjected to clino-rotation, inversion, and static 1 G environments. Different cell densities (confluent and sparse), not previously considered, were investigated, and inverted cultures were examined in addition to those continuously changing with respect to the direction of the applied vector of gravity (clino-rotation). Interestingly, statically inverted and dynamically rotated (clinostat) cultures exhibited similar results for both confluent and sparse cultures.

Materials and methods

Cell culture

Osteoblasts were isolated from the calvaria of 17-day-old chicken embryos (Spafas Inc., Norwich, CT) by three trypsin-collagenase digestions as previously described (Gerstenfeld et al. 1987). Cultures were grown in Minimal Essential Medium Eagle (MEM) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Chemical Co., St. Louis, MO), in 100 mm culture dishes, until confluence was achieved (10–14 days). The medium was changed 3 times per week. Upon reaching confluence, cells were trypsinized and reseeded (500,000 cells) in 100 mm culture dishes each of which contained 10 type I collagen-coated coverslips 22 mm × 12 mm in size. Osteoblasts attached to coverslips were initially grown in MEM + 10% FBS. Subsequently, two types of cultures, *sparsely plated* and *densely plated* were produced for the experiments.

In experiments using *sparsely plated* cells, the

coverslips with attached osteoblasts were placed directly into Fluid Processing Apparatus (FPA) units (see below) containing 3.5 ml of MEM + 10% FBS approximately 12–36 h after the coverslips were seeded ($\sim 10,000$ cells cm^{-2}). Some day 0 coverslips were fixed as baseline controls prior to inversion or clino-rotation.

For experiments using *densely plated* coverslips (confluent), the medium in these dishes was changed every other day for 1 additional week of growth. After the achievement of confluence on coverslips, the medium was changed to Dulbecco's Modified Eagle's Medium (DME) with 10% FBS. The next day the medium was replaced with DME + 10% FBS supplemented with 10 mM β -glycerophosphate. A day later the replaced medium was additionally supplemented with $12.5 \mu\text{g ml}^{-1}$ ascorbate, the total combination is considered 'complete DME'. The following day the coverslips with attached osteoblasts (mostly in the quiescent phase, G_0 , as the cells had been confluent for ~ 4 days) were removed from culture dishes and were loaded into FPA units containing 3.5 ml of 'complete DME' ($\sim 275,000$ cells cm^{-2}).

Apparatus

To simulate conditions osteoblasts would encounter in spaceflight, each coverslip was placed in a completely filled fluid compartment of a Fluid Processing Apparatus (FPA). The FPA was designed for use on the Space Shuttle by BioServe Space Technologies, Boulder, Colorado, USA (Luttges 1992). The FPA, depending on the specific protocol design, allows experiments to be initiated and terminated in flight through chemical fixation of living specimens. Figure 1 presents a schematic of the FPA and describes its operation and contents as adapted for the experiments of this study. For these experiments 3.5 ml of medium was loaded into the chamber of the FPA. This was the required amount of medium to hold the coverslip between the rubber septum and gas exchange membrane so that the coverslip would not move within the FPA.

The clinostat rotates in 2 dimensions at 1.5 rpm on the long axis and 2.3 rpm radially (Smith et al. 1999; Kacena 1999). This rotation continuously changes the direction of gravity applied to the coverslips. Experiments were performed at 37 °C, 5% CO_2 , and 95%

Figure 1. The Fluid Processing Apparatus (FPA), a spaceflight-qualified apparatus, as specifically utilized for osteoblasts grown on coverslips in the laboratory. The FPA allows experiments with living cells to be terminated in space. In this configuration the FPA has 1 chamber which contains a type I collagen coated coverslip with attached osteoblasts, surrounded by 3.5 ml of 'complete DME' (densely plated cultures) or MEM (sparsely plated cultures).

relative humidity with all samples in the same tissue-culture incubator.

Experimental procedure

Osteoblasts attached to coverslips were loaded into FPA units, and the units were oriented so that the cells were right-side up ($A \downarrow G \downarrow$ – A is the substrate attachment direction and G is the direction of the gravity vector) or inverted ($A \uparrow G \downarrow$). Both densely and sparsely plated samples were cultured for up to 6 d with samples being collected for analysis every 24 h. All experiments were performed at 37 °C, 5% CO₂, and 95% humidity. Confluent or densely plated osteoblast cultures were also subjected to clino-rotation or static 1 G (controls) for 3 h or 3 d. Sparsely plated osteoblast cultures were clino-rotated for 1, 2, 3, and 4 days.

Live/Dead assay

To examine viable cell number, osteoblasts grown on glass coverslips were removed from FPAs, placed with cells facing up ($A \downarrow G \downarrow$) in 35 mm Petri dishes or 6-well plates, and processed using the Live/Dead Assay kit as recommended by the manufacturer (Molecular Probes, Eugene, OR). Coverslips were examined on an Olympus Microscope OM-2 (Olympus Co., Lake Purchase, NY) with epifluorescence and a G520 ex/em barrier filter at a total magnification of 200 $\times$. Live cells emitted green fluorescent light while dead cells were red. From 8 individual experiments, 6–10 randomly-selected areas on each coverslip were digitally photographed (Nikon N90S camera mounted on a Kodak DCS 420 adapter, Rochester, NY) so that

the numbers of live, dead, and total cells per field (700 $\mu\text{m} \times 1000 \mu\text{m}$) could be counted and recorded.

Biochemical assay (alkaline phosphatase, crystal violet, and alizarin red)

For biochemical analysis of alkaline phosphatase (APase) production, viable cell number, and bound calcium, the one-dish assay for osteoblast proliferation and differentiation (Kostenuik et al. 1997) was modified for use with coverslips recovered from FPAs. Specifically, for this assay, coverslips with attached osteoblasts were removed from FPA units and placed in 6-well dishes. Then the coverslips were processed as described for a 6-well plate (Kostenuik et al. 1997).

BrdU incorporation

Cells incubated with BrdU will incorporate the BrdU if they are actively synthesizing DNA (in S-phase of the cell cycle). Therefore, to examine the percentage of cells in S-phase, a previously published protocol was modified for use with FPA cultures (Gerstenfeld et al. 1998). For these experiments, each FPA was injected with 70 μl of BrdU solution (6.0 mg BrdU (5-Bromo-2'-Deoxyuridine, Sigma Chemical Co., St. Louis, MO)/1 ml media). Cultures were gently inverted and rotated 3 times to facilitate mixing. Any air bubbles formed were removed using a 23 G needle. Cultures were then returned to the appropriate configuration (right-side up, inverted, or clinostat). All FPAs were processed within approximately 5 min. Cultures were then incubated with BrdU for either 2 or 24 h. The 24-h incubation time was selected to see how many total cells were actively proliferating in the cultures since these cells double every 18–28 h. The 2-h incubation time was chosen to represent the percentage of cells in S-phase at the given time point. After incubation, coverslips with attached cells were carefully removed from FPAs and placed in 6-well dishes where they were processed for fluorescent antibody labeling as previously described (Gerstenfeld et al. 1998). After processing, coverslips with attached cells were examined by fluorescence microscopy with a G520 ex/em barrier filter (200 $\times$).

Enumerated fluorescent cells were those that entered or were in S-phase of the cell cycle at any time during BrdU exposure. Total cell number was de-

terminated by examining, under phase contrast, the same field in which fluorescent nuclei were counted.

Results

In order to determine whether static or dynamic directional alterations affect the attachment of osteoblasts in confluent and sparsely plated cultures, the number of attached cells was counted in light micrographs or determined biochemically using crystal violet dye. Additionally, proliferation was assessed by BrdU incorporation assays, and cell function was assessed by analyzing alkaline phosphatase production and bound calcium.

Densely plated cultures

Number of attached cells

The number of attached osteoblasts present in densely plated cultures that were right-side up ($A\downarrow G\downarrow$) and inverted ($A\uparrow G\downarrow$) was determined from light micrographs (Live/Dead Assay) as well as crystal violet assay measurements. Figure 2A is a representative graph depicting the number of viable cells over a 6 d experiment (to confirm reproducibility, 4 separate experiments were performed). For the Live/Dead Assay experiments (as shown in Figure 2A), the cell number data represents the number of attached osteoblasts counted in 6 microscopic fields in ~6 separate samples. The data indicate that there was no significant difference (majority of time points) between right-side up and inverted samples in any of the analyses, in terms of number of total or viable attached cells. Microscopic cell counts were also performed on clino-rotated and control samples. For these 4 experiments only 2 time points were analyzed (a clinostat can hold only 8 FPAs), 3 h and 3 d. The data (not shown) from these experiments also indicated that there was essentially no significant difference in viable cell number among clinostat and control cultures. However, in one of the 4 experiments there were significantly more cells in the 3 d clinostat cultures than simultaneous control cultures. Since this result was not reproduced in 3 additional clino-rotation experiments and was not seen in all 4 of the inversion experiments, it is suggested that when confluent osteoblasts are already well attached to a substrate their attachment and viability are not affected by changes in gravitational direction.

Figure 2. Osteoblast cell number in densely seeded (A) and sparsely seeded (B), normal, inverted, and clinostat cultures as determined in Live/Dead Assay experiments.

Figure 3. Time course of alkaline phosphatase production/cell in densely seeded (A) and sparsely seeded (B), normal and inverted cultures.

Function of attached cells

Confluent osteoblast cultures were examined for alterations in alkaline phosphatase and bound calcium. These biochemical analyses showed that there was no

Figure 4. Time course of bound calcium/cell in densely seeded, normal and inverted cultures. The ratio of these dyes is proportional to bound cellular calcium.

significant difference in alkaline phosphatase or precipitated calcium levels in inverted cultures compared to simultaneous 1.0 G controls (Figures 3A and 4 are representative of the 2 separate experiments). Interestingly, as Figure 4 illustrates, bound calcium levels in inverted and 1.0 G cultures significantly increased on day 3, remained elevated on day 4, and returned to initial levels on days 5 and 6. As these experiments were optimized to attempt to catch peak levels of bound calcium, as well as alkaline phosphatase, in confluent, differentiating cells, the elevated levels on days 3 and 4 are not surprising since they actually represent 13 and 14 days of culture, respectively. Further, it is thought that the decline on days 5 and 6 is a result of acidic degradation of bound calcium as phenol color changes suggest a more acidic environment at these later time points (cultures were not fed during the 6 day experimental period). Overall, these alkaline phosphatase and bound calcium results suggest that the function of osteoblasts in confluent cultures were not adversely affected by changes in gravitational direction.

Sparsely plated cultures

Number of attached cells

Although attachment of confluent, primarily differentiating osteoblasts appeared unchanged when culture orientation was altered with respect to gravity, it was hypothesized that osteoblast attachment may be altered in proliferating cultures, especially since cells reduce attachment sites as they round during mitosis. Results from Live/Dead Assay experiments (Figure 2B), BrdU incorporation experiments, and crystal violet assay experiments were consistent, and all show that there was a significant decrease in the number of total and viable cells in sparsely plated

inverted and clinostat cultures compared to controls at 1 d, 2 d, and occasionally 3 d. However, after day 4 there was no significant difference among experimental conditions. It should be noted that the graph is of the number of viable cells, although it is essentially the same as the number of total cells as over 97% of attached cells were viable. For the number of attached osteoblasts in sparsely plated cultures to be lower initially and then to 'catch up' by day 4 it would be necessary for the exponential growth rate (μ) to be higher for a period of time in clinostat and inverted cultures, or the lag phase would have to be shorter in control cultures. Table 1 shows the exponential growth rates for the sparsely plated osteoblast cultures during the exponential phase. The growth rate data suggest that there is a longer lag phase in inverted and clino-rotated cultures since the inverted and clino-rotated growth rates were slightly higher (not significant) than control growth rates. Further, the inverted and clinostat growth curves (which are essentially identical) appear to be shifted to the right of control curves by approximately 1 d. This is more evidence of an increase in lag-phase duration in inverted and clinostat cultures. In summary, the culmination of these data suggest that altering the cell-substrate attachment direction with respect to the gravity vector ($G\downarrow$) causes an immediate response in diminishing total and viable attached osteoblast number in sparsely plated, proliferating osteoblast cultures, but the effect disappears after this initial time period.

Function of attached cells

In addition to cell number, alkaline phosphatase levels were measured in right-side up and inverted cultures. Representative results from these experiments are given in Figure 3B. The data show that there was essentially no significant difference between right-side up and inverted samples at any time point (when normalized per cell). This was especially evident when taking into consideration the levels seen in the confluent cultures (Figure 3A), where the beginning levels are equal to or exceed the levels seen in these sparsely-plated-cell experiments. This result suggests that APase production/cell remains unaffected by inertial vector direction for the entire time course of these experiments.

Proliferation of attached cells

To analyze further the kinetics of sparsely plated attached cells, the percentage of cells undergoing

Table 1. Exponential growth rate (μ) of sparsely plated osteoblast cultures grown under various gravitational conditions

Assay	Experiment Number	Gravitational Condition	μ -value
Live/Dead	1	1.0 G	0.24 ± 0.05
		Inverted	0.31 ± 0.12
	2	1.0 G	0.20 ± 0.04
		Inverted	0.34 ± 0.12
	3	1.0 G	0.34 ± 0.08
		Inverted	0.43 ± 0.05
	4	1.0 G	0.34 ± 0.10
		Inverted	0.28 ± 0.16
	5	Climo-rotated	0.33 ± 0.10
		1.0 G	0.37 ± 0.10
	6	Inverted	0.37 ± 0.16
		Climo-rotated	0.39 ± 0.10
BrdU	1	1.0 G	0.30 ± 0.08
		Inverted	0.38 ± 0.19
		Climo-rotated	0.34 ± 0.08
	2	1.0 G	0.28 ± 0.05
		Inverted	0.35 ± 0.10
		Climo-rotated	0.35 ± 0.10
	3	1.0 G	0.29 ± 0.02
		Inverted	0.34 ± 0.05
		Climo-rotated	0.34 ± 0.05
	4	1.0 G	0.32 ± 0.07
		Inverted	0.36 ± 0.06
		Climo-rotated	0.34 ± 0.05
Crystal Violet	1	1.0 G	0.23 ± 0.03
		Inverted	0.33 ± 0.09
	2	1.0 G	0.30 ± 0.04
		Inverted	0.33 ± 0.07
	3	1.0 G	0.32 ± 0.09
		Inverted	0.38 ± 0.10

There were no significant differences among samples

Figure 5. Percent of cells in S-phase with 2 hr and 24 hr BrdU labeling in sparsely seeded, normal, inverted, and clinostat cultures. Upper curves: percent proliferating cells; lower curves: percent S-phase cells.

proliferation was assessed by BrdU incorporation as described above. Following a 24 h BrdU exposure nearly all cells were labeled ($\sim 94\%$), a result indicating that virtually every attached cell was proliferating (entering S-phase) throughout the experiments under all conditions. Results also indicated that proliferation was essentially identical in the first 2 days and that by day 4 the percentage of cells in S-phase (2-h label) decreased in right-side up cultures compared to clinostat and inverted cultures (Figure 5 – representative graph from 4 separate experiments). This decline between days 3 and 4 in the percentage of S-phase cells in controls would suggest an approach into stationary phase, whereas the labeling index did not decline in rotated and inverted cultures, which, according to growth curves, entered stationary phase later than did control cultures.

Discussion

To better understand the influence of gravity on cells in culture, several investigators have rotated cells in clinostats. Here we used both clino-rotation and inversion to alter the direction of gravity with respect to substrate attachment. Interestingly, we found virtually identical results in terms of osteoblast number, growth rate, percentage of S-phase cells, APase production, and bound calcium in clino-rotated and inverted cultures. This may suggest that a less complicated model for spaceflight (inversion) may be applicable for some experimental measurements using attached cells, such as osteoblasts.

As described below, some research has shown alterations with the degree of attachment or maturity prior to rotation, and alterations with exposure duration. Gruener and Hoeger (1990) found that nerve and muscle cells in contact may form nerve-associated acetylcholine receptor patches (NARPs). If co-cultures were rotated on the clinostat before any NARPs were formed, the number of NARPs was significantly reduced compared to controls. If rotation began after established contact (many NARPs already formed), the number of NARPs was unchanged. These data seem to suggest that the degree of cell maturity or attachment may be a critical factor in cell-gravitational responses *in vitro*. This conclusion with nerve-muscle co-cultures is consistent with the data in this study for osteoblasts. Confluent cultures of osteoblasts, which principally differentiate as opposed to

proliferate, were less affected by modified inertial environments, than were sparse osteoblast cultures.

With respect to exposure duration, one laboratory in particular has examined the effects of clino-rotation on the cytoskeletal structure of eukaryotic cells. Gruener (1985), Gruener and Hoeger (1990, 1991) found that muscle cell striations appeared later and were less organized than in static control cells. With 24 h of rotation approximately 50% of the clino-rotated cells were striated whereas 75% of control cells were striated. However by 72 h there was no difference in the percentage of striated cells. Such temporal trends may suggest that certain cell functions are initially inhibited or delayed by clino-rotation but that with time those functions recover, possibly by cells adapting to their environment. This 'catching up' was also exhibited in the present studies, in which sparsely plated cultures had fewer cells in the first few days of the experiment, but by day 4 no differences in cell number were found between treated cultures and controls. The recovery of the cells was further demonstrated by BrdU labeling, where inverted and clino-rotated cell cultures had a higher percent of cells in S-phase in the latter period of the experiment (days 3–4), a result consistent with cell numbers determined by the end of day 4.

In summary, for confluent or densely plated osteoblast cultures, the number, viability, and function of attached cells were not altered by changes in the direction of cell attachment relative to gravity. While there appears to be no significant difference in studied parameters for confluent, non-proliferating cultures exposed to clino-rotation or inversion, there were differences in sparse, highly proliferative cultures. Within the first 24 h after sparsely plated osteoblasts (heterogeneous mix of cell cycle phase) were placed in an altered gravitational environment, there were significantly fewer total attached cells. The initially low cell number was corrected by days 3 or 4 of growth, probably owing to shortened lag phase durations as opposed to a heightened growth rates. Finally, when normalized per cell, alkaline phosphatase production was nearly identical between experimental and control cultures and from one collection time to the next, suggesting normal osteoblast function *in vitro*. Therefore, it appears that even though changing cell-substrate attachment direction with respect to the gravity vector causes an immediate response in diminishing viable osteoblast number in sparse cultures, the effect disappears with time and all attached cells function similarly.

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