Effect of Microgravity on Osteoblast Growth Activation: Analysis of Transcription, Translation and Morphology in ESA Biorack Flight STS-76

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The unique environment of microgravity can place unusual stress on, and cause many physiological changes in organisms that evolve in a 1g (normal gravity) environment [1-3]. Some of the basic physiological changes in man include loss of fluids and electrolytes, muscle atrophy, space motion sickness, anaemia, reduced immune response, and significant loss of calcium and mineralised bone. The continuous and progressive loss of calcium and weight bearing bone noted in space flight crews is one of the most serious impediments to long duration manned space flight. Skeletal changes and loss of total body calcium have been noted in both humans and animals exposed to microgravity from between 7 and 237 days. During the American Apollo and Skylab missions, photon absorptionmetry was used to assess pre- and post-flight bone mineral mass. For the 12 crew members of Gemini 4, 5, and 7, and Apollo 7 and 8, the average post-flight loss from the Os Calcis (heel) was 3.2% over an average of 8.5 days [3,4,5,6].

A direct effect of microgravity is the loss of mechanical stress on the skeletal system. Although in-flight exercise has proven to be a helpful countermeasure used by both the American and Soviet space programs, the greatest losses in the United States flight programme occurred in the 84-day Skylab 4 mission where exercise was regularly performed. Crew members using exercise as a countermeasure still lost an average of 4% of bone over the 84 day mission period [7]. In the 237 day Soviet Soyuz T-10 mission, the Cosmonauts lost bone in spite of two to four hours of daily exercise. Both compact and trabecular bone were lost from the Os Calcis during this mission. Bone loss appears to increase in general proportion to mission length, from 4 percent to 19.8% over an 84 to 184 day period [8,9]. The loss of bone in the presence of consistent exercise suggests additional molecular mechanisms are responsible for bone loss.

Several lines of evidence, from both human and animal studies, have demonstrated that the bone loss occurring in space flight is due to a decrease in bone formation. The decrease in bone formation and osteoblast growth is likely to be due to both direct and indirect (systemic) effects of microgravity. The early stages of growth activation require gene expression and transition from a G1 state to DNA synthesis. In these Biorack studies, we have used the MC3T3-E1 osteoblast as a bone model to study the direct effects of microgravity on activation of quiescent osteoblasts and the molecular mechanisms which regulate decreased osteoblast growth in space flight.
Materials and methods

Flight instruments

Biorack is a multi-user facility and consists of incubators with variable gravity centrifuges, a cooler, a deep freeze and a sealed glove box. Two identical Biorack models were used: one Ground Model remained on Earth, the other was flown in Spacehab on the Space Shuttle. Biorack has an important advantage over other microgravity facilities in that is provides a small radius (78 mm) slow rotating (107.0 ±0.5 rpm) centrifuge (Figure 1). The centrifugal force results in a 1xg on board control. Because of proximity, both the 0xg (zero gravity) and 1xg samples experience identical launch vibrations, accelerations, cosmic radiation and other unknown conditions in the flight. The only difference between the flight groups is the gravity parameter.

Experimental flight hardware was designed according to ESA specifications for use in the Biorack facility and was constructed by Centrum voor Constructie en Mechatronica (NL). The hardware consisted of the CCM plunger box and its black Type I container (Figure 2) developed for space flight cell culture. The Type I container provided a second level of fluid containment. The plunger boxes were designed to provide a sterile environment for cell growth activation and fixation in a microgravity condition. The plunger-box is composed of two culture chambers, each of which holds two 11x22 mm glass cover slips and is connected to three cylindrical compartments filled with media or fixative. The medium is forced into the culture chamber by releasing a spring load plunger by sequential manual activations that allow medium, fixative liquid changes. The sealed plunger boxes allow astronauts to activate and inactivate cell growth in space flight and store the samples for later analysis on Earth. There are six plunger reservoirs, four that hold the activation media (labelled A, B, D, E). Activation of the quiescent cells was accomplished by activation of media reservoir A and C. The media in chambers B and E were back-up in case the first chambers malfunctioned. The reservoir (labelled C, F) hold a special solution of guanidinium thiocyanate (GITC) solution for preservation of RNA (ribonucleic acid) or proteins. For cell morphology studies, the preservation solution was formaldehyde. The samples fixed in GITC solution were stored at −20°C and the formaldehyde fixed samples held at 5°C.

Statistics

Mean and standard deviation were calculated for all parameters. Group differences were assessed by unpaired t-test, the significant levels were P<0.05 or less.

Osteoblast culture and analysis:

Cell culture

Each plunger box container has two cell chambers each holding two cover slips. Cells used in this work were mouse MC3T3-E1 osteoblasts which were clonally derived from embryonic mouse calvaria [10]. The cells were grown in T-75 flasks with 10% foetal
bovine serum-containing a-MEM medium supplemented with L-glutamine, HEPES (N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]) and antibiotic-antimycotic solution (containing penicillin, streptomycin and amphotericin B) as previously described [11]. Cells were maintained at a subconfluent density in a 37°C incubator with 5% CO₂ and fed or split three times a week. For each experiment, 120,000 to 200,000 cells were plated into each 11 x 22 mm cover slip (Thomas Scientific, NJ), placed in 6-well plates, and grown in 10% serum-containing medium overnight. Cell-coated cover slips were transferred into the plunger box units in 2% foetal bovine serum containing medium for flight. As a result of the interplay between planned schedule for activating Biorack and the deadlines for handing over samples, the units were held 19 h in the Shuttle at mid-deck temperature prior to launch and for 18 h in microgravity.

Activation
At the time of activation, the cells were quiescent since they had been incubating for 37 h at Shuttle cabin temperature in media containing 2% FCS (foetal calf sera). This ensured that any changes in mRNA levels are due to the sera activation in space flight and not to previous levels of growth factors or other components in the media. The 1xg flight osteoblast controls were exposed to acceleration on the centrifuge of the Biorack 1xg incubator. Each experiment had four replicates for each time point. Time of activation and deactivation are shown in Figure 3.

RNA isolation, reverse transcription and PCR
RNA from cultured MC3T3-E1 osteoblasts were isolated using a modified guanidinium thiocyanate method (manuscript in preparation). This method is based on the protocol previously described by of Chomczynski and Sacchi [12] and polymerase chain reactions (PCRs) were performed and the PCR products analysed as previously described [13].

Protein isolation and quantification
Protein, remaining in the guanidinium thiocyanate solution after the RNA extraction, was isolated from each sample by a modified method previously described by Chomczynski. Protein was quantified by DC Protein Assay (Bio-Rad Laboratories, CA) according to the manufacturer’s protocol and read on a DynaTech 96-well plate reader. The standard curve and protein quantification results were interpreted with BioLinx software.

Cell morphology
Cover slips were fixed in 3.7% formaldehyde in phosphate buffered saline during flight and stored at 4°C. Cover slips were stained for 30 minutes with 4 units/ml rhodamine-phalloidin (Molecular Probes, Inc., OR) and 3 µg/ml Hoechst dye (Calbiochem, San
Diego, California). Upon return to Earth, the preserved cell nuclei were stained with Hoechst 33258 (Sigma, MO) as previously described [4] and F-actin was stained with rhodamine phalloidin (Molecular Probes, OR). Dried cover slips were mounted onto slides and photographed under a Zeiss Axioscope microscope at 10, 40 and 100x magnification. Slides were scanned and digitised for computer analysis.

Results

Pre-flight results

These pre-flight experiments have been published [13,14] and are summarised here to put other data into context. Both pre-launch experiments demonstrate the importance of the Biorack on board 1xg control.

Gravity effects on gene expression

It is apparent to those who have flown on the Space Shuttle that launch forces are severe enough to cause changes in metabolism of biological systems. Serum-deprived mouse osteoblastic cells (MC3T3-E1) were centrifuged at room temperature under a regime designed to simulate a Space Shuttle launch (maximum of 3xg). mRNA levels for nine genes involved in bone growth and maintenance were determined using RT-PCR. 30 minutes after centrifugation the mRNA for early response gene, c-fos, was significantly increased 89% (P<0.05) with respect to the non-centrifuged control samples. The c-fos induction was transient and returned to control levels after three hours. mRNA for the mineralisation marker gene, osteocalcin, was significantly decreased to 44% of control levels (P<0.005), three hours after centrifugation. No changes in mRNA levels were detected for c-myc, TGFb1, TGFb2, cyclophilin A, or actin. In addition, no change in the steady state synthesis of prostaglandin E2 (PGE2) was detected, possibly due to lack of lipid substrates in serum deprived cells. This suggests that the increase in c-fos mRNA in response to gravitational loading is a result of mechanical stimulation. These results indicate that a small magnitude mechanical loading, such as that experienced during a Shuttle launch, can alter mRNA levels in quiescent osteoblastic cells [13].

The effect of vibration on gene expression

Serum-deprived mouse osteoblastic (MC3T3-E1) cells were subjected to vibration forces designed to simulate a Space Shuttle launch. The mRNA levels for eight genes were investigated to determine the effect of vibration on mRNA expression. Two growth-related protooncogene, c-fos and c-myc, mRNA levels were upregulated significantly within 30 minutes following vibration, while those of osteocalcin as well as the transforming growth factor-β1 were decreased significantly within three hours following vibration.

No changes were detected in the levels of β-actin, histone H4 or cytoplasmic phospholipase A2 following vibration. No basal levels of cyclooxygenase-2 expression were detected. In addition, the extra-cellular concentrations of PGE2, a potent autocrine/paracrine growth factor in bone, were not altered significantly following vibration. The lack of changes in the PGE2 released was probably due to the serum deprivation state of the osteoblasts. In comparison to the gravitational launch profile, changes in gene expression due to vibration was greater both in magnitude and in the number of genes activated. These data suggest that the changes in mRNA expression is due to a direct mechanical effect of vibration on the osteoblast cells and not due to changes in the local PGE2 concentrations [14].

In flight results

RNA analysis

Details of the osteoblast RNA content 3 h after sera activation is seen in Figure 4. The total RNA isolated at 3 h after activation in on-ground, 0xg and 1xg conditions (n=4 for each condition) did not significantly change in the microgravity condition.
The analysis of the morphology of cells grown in on-ground (OG) and 1xG conditions.

Figure 6. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 7. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 8. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 9. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 10. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 11. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 12. Cytoskeletal E1Rin in conditions a and 1xG.

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Figure 95. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 96. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 97. Cytoskeletal E1Rin in conditions a and 1xG.

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Figure 99. Cytoskeletal E1Rin in conditions a and 1xG.

Figure 100. Cytoskeletal E1Rin in conditions a and 1xG.
demonstrate a change in cell cytoskeleton and nuclear architecture (Figure 6). After being in microgravity, the 0xg cells and their nuclei elongate. The cell shape of the 1xg osteoblast and its nuclei is rounded and several mitotic figures are present.

**Discussion and conclusions**

Numerous studies have suggested that microgravity influences cell growth and differentiation [1,2,3,11,15,16]. Cell growth occurs in four states, G1 where early gene activation occurs, S-phase where active DNA synthesis occurs, G2 where DNA content has been doubled and is preparing to divide into daughter cells, and mitosis where the cell divides into the two daughter cells. It is possible that the down-regulation of cell growth is due to a general toxicity in space flight caused by unfavourable culture conditions including, effects of launch, lack of gravity or increased cosmic radiation.

Our findings show that the 0xg and 1xg cells are actively synthesising RNA and protein in space flight. This data is based on analysis of RNA extracted from the cells three hours after activation, the presence of multiple nucleoli 27 h after activation and absence of apoptotic chromatin condensation. There was no significant change in the RNA content of the ground, 0xg and 1xg cells. Fluorescent staining of the nuclei demonstrated the absence of apoptosis and the presence of active RNA synthesis in multi-point nucleoli in the ground, 0xg and 1xg cells. A significant difference did appear in the architecture of the nuclei with the 0xg nuclei becoming elongated after activation of the quiescent cells. The 0xg cell cytoskeleton also showed elongation of the cell morphology to a spindle type structure.

It has been demonstrated that disruption of cell cytoskeleton in anchorage dependent cells can cause inhibition of cell growth. The causes for cell shape change under microgravity conditions is now under investigation. Finally, in the 1xg samples exposed to gravity for 27 h, there was an apparent increase in the number of G2 and mitotic cells when compared to the 0xg osteoblasts.

In addition to the 1xg onboard control, a second important part of this investigation is the fact that we had multiple samples and were therefore able to get statistics on the flight experiment. Since a decrease in cell growth could be caused by a decrease rate of transcription, we analysed the overall rate of RNA synthesis in the ground, 0xg and 1xg samples. When RNA was isolated, we found that there was no significant change in the overall RNA synthesis during the first three hours of growth activation.

We were also able to devise methods that allowed us to examine total protein in the same samples from which we isolated the RNA. Our findings show a significant reduction in total protein translation in both the 0xg and 1xg flight samples. Both flight samples were exposed to continuous microgravity during the 17 h of flight before activation. The presence of gravity for the three hours of activation allowed the total protein in the 1xg samples to increase, but the data was not significant when compared to the 0xg samples. Future analysis of other OSTE0 samples exposed to 1xg for longer periods will allow us to determine if microgravity is causing a permanent change in total protein synthesis.

In conclusion, this study demonstrates that microgravity does not affect early RNA induction associated with sera activated growth. Our second finding is that early protein synthesis was significantly reduced suggesting a microgravity effect on early protein translation. In addition, cells in 0xg had a dramatic change in cell morphology and nuclear architecture which may play a role in reduced growth of osteoblasts in space flight. Finally, these studies show the importance of having on-board 1xg control to assess the actual role of microgravity on living systems.
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References


