

CHANGES IN GENE EXPRESSION AND SIGNAL TRANSDUCTION IN MICROGRAVITY

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Abstract

Studies from space flights over the past three decades have demonstrated that basic physiological changes occur in humans during space flight. These changes include cephalic fluid shifts, loss of fluid and electrolytes, loss of muscle mass, space motion sickness, anemia, reduced immune response, and loss of calcium and mineralized bone. The cause of most of these manifestations is not known and until recently, the general approach was to investigate general systemic changes, not basic cellular responses to microgravity.

This laboratory has recently studied gene growth and activation of normal osteoblasts (MC3T3-E1) during spaceflight. Osteoblast cells were grown on glass coverslips and loaded in the Biorack plunger boxes. The osteoblasts were launched in a serum deprived state, activated in microgravity and collected in microgravity. The osteoblasts were examined for changes in gene expression and signal transduction. Approximately one day after growth activation significant changes were observed in gene expression in 0-G flight samples. Immediate early growth genes/growth factors *cox-2*, *c-myc*, *bcl2*, *TGF β 1*, *bFGF* and PCNA showed a significant diminished mRNA induction in microgravity FCS activated cells when compared to ground and 1-G flight controls. Cox-1 was not detected in any of the samples. There were no significant differences in the expression of reference gene mRNA between the ground, 0-G and 1-G samples. The data suggest that quiescent osteoblasts are slower to enter the cell cycle in microgravity and that the lack of gravity itself may be a significant factor in bone loss in spaceflight. Preliminary data from our STS 76 flight experiment support our hypothesis that a basic biological response occurs at the tissue, cellular, and molecular level in 0-G. Here we examine ground-based and space flown data to help us understand the mechanism of bone loss in microgravity.

Introduction

Many physiological adaptations to microgravity have been documented in humans (1-3). These changes include increased levels of glucocorticoids, loss of muscle (up to 25 percent loss of wet muscle weight), reduced immune function (immune cells fail to be activated by

mitogen), and significant loss of calcium and bone caused most probably by reduction of osteoblast growth.

Some of the physiological changes in bone loss in spaceflight maybe due to systemic or hormonal changes in the body; however, many are probably a direct effect of low mechanical stress at the cellular level. The loss of bone, sometimes as high as 1% per month, is one of the major physiological 'show stoppers' in planning a 30-month manned mission to Mars. Here we discuss our data from osteoblasts in ground based and flight experiments from Shuttle missions.

We have previously noted reduced growth of osteoblasts in spaceflight and most recently have noted significant changes in the ability of the osteoblasts to be activated to grow in spaceflight. The slowing of cell growth in microgravity is accompanied by a significant reduction in induction of several immediate early gene messages RNA as well as an uncoupling of prostaglandin signal transduction in growing osteoblasts.

Methods

Preparation of Osteoblasts: The early passage osteoblast cell line was kindly provided to us by Dr. M. Kumegawa (Josai Dental University, Japan) and cells grown as previously described (4).

Measurement of PGE₂: A competitive enzyme immuno-assay (EIA, Cayman Chemical, Ann Arbor, MI) was used to assess the PGE₂ levels in the osteoblast media and has been previously described (5, 6).

Experimental hardware: Flight experimental hardware was designed by Centrum voor Constructie en Mechatronica (Nuenen, the Netherlands) for our experiment, "OSTEO," which was flown as part of the Biorack payload on space shuttle flights STS-76, 81 and 84. The hardware consists of two separate units: the plungerbox unit type HM 2/3 and the Type I Container. The plungerbox unit contains two cell chambers each holding two 22 x 11 mm glass coverslips on which the cells grow. The plunger box is placed into a type I container, which provides a secondary containment for biological samples during the experiment. Each RNA sample was isolated from the cell lysates of two coverslips and represents one data point, total n=4.

RNA isolation: RNA was extracted and purified by a modified acid guanidium thiocyanate/phenol/chloroform extraction method. RT-PCR analysis- The semi-quantitative RT-PCR analysis and primer selections have been described previously (4, 7-9). To correct for small variations between experiments, each PCR product was compared to an internal standard of cyclophilin or 18S products derived from the same RT reaction. Since sample size was small (200,000 cells) RNA content was held constant and linear RTPCR was accomplished by varying the number of PCR cycles. PCR conditions were established so that amplification reaction was stopped in the linear range. FCS activation occurred in orbit approximately 19 hours after launch. All data were derived using semi-quantitative RTPCR from triplicate or

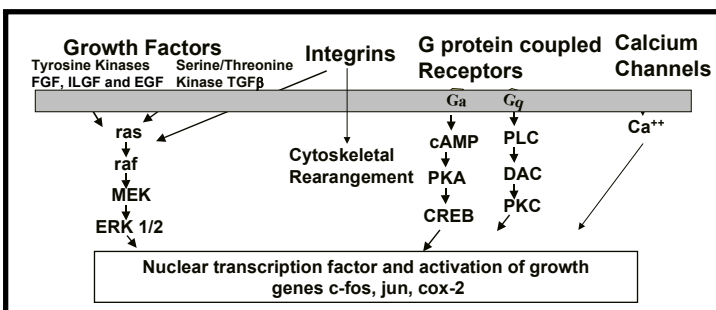
quadruplicate samples. Cyclophilin or 18S mRNA expression was used to normalize expression of the genes.

Results and Discussion

Changes in gene expression caused by low gravity forces: Since life on Earth evolved in a 1-G environment, it was our hypotheses that gravity itself may have an effect on bone growth. Our preflight data focused on the changes in growth that may be due directly to the lack of gravity. In these ground experiments, we grew the MC3T3-E1 osteoblasts on coverslips placed in Biorack plunger boxes in a serum deprived state as we did on the shuttle (STS-76) experiments. The cells were subjected to 3-G gravitational forces for 8 minutes. It is known that PGE₂ and PGI₂ are released during exercise, therefore evoking the thought that the same pathway may be implicated in bone loss occurring in microgravity.

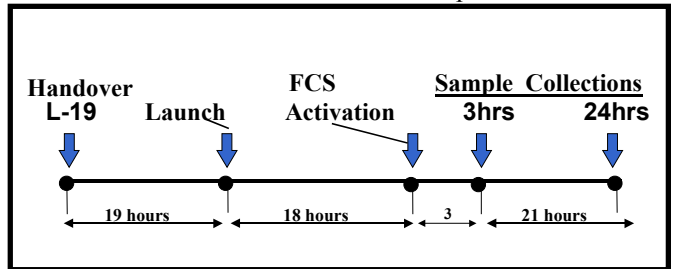
Low levels of gravity for a short period cause changes in gene expression: Serum-deprived mouse osteoblastic cells (MC3T3-E1) were centrifuged under a regime designed to simulate a Space Shuttle launch (maximum of 3g). mRNA levels for 9 genes involved in bone growth and maintenance were determined using RT-PCR. 30 minutes after centrifugation the mRNA for early response gene, *c-fos*, was significantly increased 89% (P<0.05). The *c-fos* induction was transient and returned to control levels after 3 hours. mRNA for the mineralization marker gene, osteocalcin, was significantly decreased to 44% of control levels (P<0.005), 3 hours after centrifugation. No changes in mRNA levels were detected for *c-myc*, TGFβ1, TGFβ2, 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 (7). Changes in gene expression due to mechanical force of gravity can be caused by several components of the cell. The mechanisms by which mammalian cells respond to gravitational signals are still unknown. However, we know that there are several mechano transducers seen in Figure 1 in the cell that may be responsible.

Figure 1. Possible pathways for mechanotransducers



Changes in osteoblast gene expression in flight

The majority of cell culture experiments sent into space have been actively growing at the time of launch. Moreover, lack of refrigeration has previously limited onboard sample preservation and therefore many experiments were terminated after landing on Earth. Even for the few experiments that had the opportunity for onboard collection, the data was compromised by flight imposed limitations including: lack of sufficient sample numbers, addition of supplements directly affecting gene expression, lack of fresh media changes and lack of onboard 1-G controls. In order to obtain our goal to study growth activation in microgravity, we launched cells that were in a quiescent condition and were not sera activated until on-orbit in the microgravity environment. There were four samples for each time point in 0-G and 1-G flight samples. Refrigerator and freezer space was available for reliable sample storage. The timeline for timeline of handover launch and sample collection is



shown in Figure 2.

Figure 2: Timeline for Osteo experiments

In contrast to previous space flight experiments, media supplements such as dexamethasone, β-glycerol phosphate and ascorbic acid were not added to our media since these agents are known to directly effect gene expression and cell morphology in the growing osteoblast.

Gene Expression:

Several reports have described gravity-specific changes in mRNA levels following exposure of cultured cells and whole animals to varying periods of microgravity in experiments performed in spaceflight, sounding rockets and clinostats. We examined quiescent cells for gravity-dependent changes in mRNA levels for 9 genes involved in bone cell growth and maturation. We asked the question: Does gravity affect the gene expression of cyclo-oxygenase-1 and 2 in flight? This is an essential question because the Cox-1 and Cox-2 enzymes are directly responsible for the synthesis of PGE₂. Since these data are preliminary, we have included a summary of significant observations. Analyses of osteoblast gene expression showed a significant diminished induction of TGFβ, bFGF, *c-myc*, *bcl2* and PCNA in microgravity. TGFβ, *c-myc* and *bcl2* expression was recovered in 1-G centrifuge flight samples. Expression of EGFr, 18S and cyclophilin were constitutive and unchanged by microgravity. The initial FCS induction of cox-2 was reduced in microgravity

although the synthesis of PGE₂ was increased in the flown 1-G samples. We found that *cox-2* mRNA is decreased in the 0-G samples when compared to 1-G controls. The copy number for *cox-1* mRNA was too low to detect. This would suggest that the cells subjected to the 0-G environment did not enter the growth phase of the cell cycle since *cox-2* is depressed in the microgravity samples.

Gene	Relative to GR 3 hours		Relative to GR 24 hours	
	0G	1-G	0-G	1-G
TGFβ	↓	=
PCNA	↓	↓
FGFb	↓	↓
BCL2	↓	↓	↓	↑
EGFr	=	=	=	=
c-myc	↓	=
Cyclin A	↓	↓	↓	↓
Cyclin E	=	=	↓	↓
Cox-2	↓	=	↓	~
cPLA2	↓	=
PGE ₂	↑	↑	↑	↑

TABLE 1: Relative expression in orbit compared to ground control. ... no samples tested; = equivalent to control; ↑ increased relative to control; ↓ decreased relative to control

Loss of coupled signal transduction in PGE₂ pathway: We have previously shown that PGE₂ is increased in flown cells after activation at early time points followed by a decrease in synthesis later in flight. At 24 hours after activation, the PGE₂ content of cells in flight is increased both in the 1-G and 0-G flown samples. The puzzling question is: why isn't the *cox-2* gene expression up regulated in the 0-G cells? Cyclooxygenase 2 is a feed forward enzyme and should be upregulated by increased levels of PGE₂ in the media. This did not happen in OSTEO microgravity flight samples. One explanation may be a change in the prostaglandin receptors in the 0-G activated cells. Previous reports have shown that the MC3T3-E1 osteoblasts respond to PGE₂ through the EP2/EP4 receptors and the PKA pathway (10).

It has been postulated that 0-G can modify receptor function, (5, 11-15). The reason for reduced growth activation by sera in microgravity is not known, however, the work of deLaat's group in other cell types has given us a basis for a hypothesis. The relationship between gene expression and microgravity has been partially described by de Laet and de Groot. They have demonstrated that a number of responses of A431 cells to epidermal growth factor (EGF) are affected microgravity. The responses include a decrease of *c-fos* and *c-jun* induction and serum response element activity (16-20). In a sounding rocket, they demonstrated that EGF-induced expression of the proto-oncogenes *c-fos* and *c-jun* were reduced almost 4 fold by microgravity. The same reduced response was seen

with serum response in microgravity. The decreased gene inductions were attributed to possible alterations in the promoter-enhancer region response. The nuclear proto-oncogenes *c-jun* and *c-fos* are known regulators of DNA synthesis (21). They have also been shown to be required for entry into S-phase DNA synthesis (22).

In later studies, the deLaat's group demonstrated that the nuclear responses to protein kinase C signal transduction were sensitive to gravity changes (17). In these studies, they demonstrated that EGF and phorbol ester (TPA) induced gene expression of *c-fos* and *c-jun* were changed by microgravity, while the calcium response was not changed, thus implicating the diacylglyceride (DAG) portion of the PKC signal transduction.

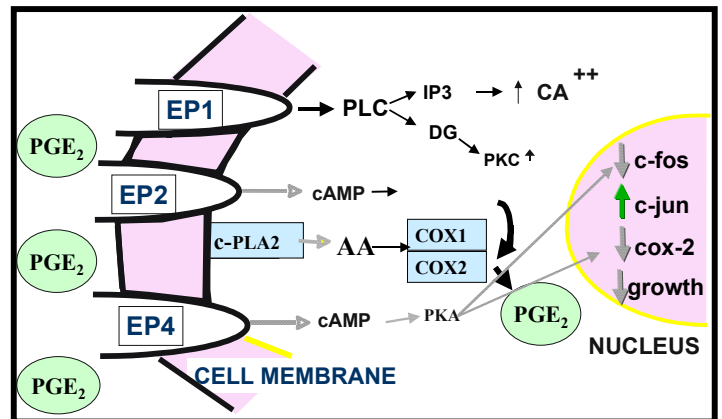


Figure 3: Microgravity induced loss of PGE₂ signaling pathway in microgravity It is possible that changes in the PGE₂ receptors could inhibit recognition of PGE₂ in microgravity. As seen in the figure above, if PGE₂ were unable to bind to the EP receptors, this would result in a high level of PGE₂ in the media and an inhibition of signaling through the 7-domain transmembrane G-coupled prostaglandin receptor pathway (shown in gray lines).

In later work, they demonstrated that EGF-induced *c-fos* and *c-jun* expression was not due to an effect on the EGF-receptor interaction since there was normal EGF-receptor redistribution in microgravity, thus suggesting that microgravity influences EGF-induced signal transduction downstream of the EGF binding and receptor redistribution, but upstream of early-immediate gene expression (23). Finally, deLaat has also demonstrated that EGF-induced actin cytoskeleton changes are affected by the arachidonic acid pathways through cyclooxygenase (prostaglandin) and 5-lipoxygenase (leukotriene) (24).

Sera activation of osteoblast growth is dependent on prostaglandin synthesis. This has been demonstrated by blocking cell growth and endogenous PGE₂ synthesis with dexamethasone and then restoring growth activity by the addition of exogenous PGE₂. PGE₂ specific induction of *cox-2* mRNA accompanied by osteoblast growth suggests that the osteoblast growth activation by PGE₂ may be required for growth. This laboratory recently demonstrated that PGE₂ induces *c-fos* mRNA via a PKA pathway (10). It is also possible that the reduction in sera-induced

osteoblast growth and cox-2 induction in microgravity may share similar mechanisms of modulation of early-immediate gene expression described by deLaat's group. It is unknown if the decrease in bone formation in astronauts is due directly to the lack of mechanical stress (or 1-G force) in microgravity, systemic changes in hormones, alterations in the signal transduction of serum growth factors, or to changes in signal transduction at the molecular level. In this paper we have shown that microgravity (lack of mechanical stress) changes prostaglandin content, response to prostaglandin, change in morphology and gene expression in osteoblasts. It is likely that decreased gravity induced mechanical stress coupled with other changes in signal transduction directly contribute to astronaut bone loss during spaceflight. It has been shown both *in vivo* and *in vitro* that blocking the prostaglandin signaling pathway can inhibit new bone formation in humans (25, 26). The resulting down-regulation of prostaglandin growth pathway in microgravity is most likely a key molecular component in the cause of space osteoporosis. In these studies we have demonstrated that microgravity causes inhibition of fetal calf sera stimulation of mRNA in 8 of 9 genes studied. Artificial gravity was able to restore message levels to normal in 5 of the 8 inhibited messages. These data suggest that artificial gravity may ameliorate the loss of bone in astronauts in spaceflight to enable mankind to go to Mars.

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