EXPERIMENTAL CELL RESEARCH 224, 103–109 (1996)
ARTICLE NO. 0116

Effects of Microgravity on Osteoblast Growth Activation

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Space flight is an environmental condition where astronauts can lose up to 19% of weight-bearing bone during long duration missions. We used the MC3T3-E1 osteoblast to investigate bone cell growth in microgravity (10^{-6} to 10^{-9} *g*). Osteoblasts were launched on the STS-56 shuttle flight in a quiescent state with 0.5% fetal calf serum (FCS) medium and growth activation was initiated by adding fresh medium with 10% FCS during microgravity exposure. Four days after serum activation, the cells were fixed before return to normal Earth gravity. Ground controls were treated in parallel with the flight samples in identical equipment. On landing, cell number, cell cytoskeleton, glucose utilization, and prostaglandin synthesis in flight ($n = 4$) and ground controls ($n = 4$) were examined. The flown osteoblasts grew slowly in microgravity with total cell number significantly reduced (55 ± 6 vs 141 ± 8 cells per microscopic field). The cytoskeleton of the flight osteoblasts had a reduced number of stress fibers and a unique abnormal morphology. Nuclei in the ground controls were large and round with punctate Hoechst staining of the DNA nucleosomes. The flight nuclei were 30% smaller than the controls ($P < 0.0001$) and oblong in shape, with fewer punctate areas. Due to their reduced numbers, the cells activated in microgravity used significantly less glucose than ground controls (80.2 ± 0.7 vs 50.3 ± 3.7 mg of glucose/dl remaining in the medium) and had reduced prostaglandin E_2 (PGE_2) synthesis when compared to controls (57.3 ± 17 vs 138.3 ± 41 pmol/ml). Cell viability was normal since, on a per-cell basis, glucose use and prostaglandin synthesis were comparable for flight and ground samples. Taken together, these data suggest that growth activation in microgravity results in reduced growth, causing reduced glucose utilization and reduced prostaglandin synthesis, with significantly altered actin cytoskeleton in osteoblasts.

INTRODUCTION

Many disruptions to the basic cellular and metabolic functions of humans and animals occur during exposure to microgravity conditions [1, 2]. Biomedical studies have shown significant physiological changes occur during space flight, which include loss of fluids and electrolytes, loss of muscle mass, space motion sickness, anemia, a reduced immune response, and a loss of calcium and mineralized bone. The progressive loss of calcium and bone may be the most critical biomedical obstacle that astronauts in extended duration space flights (e.g., Mars mission) will encounter. The microgravity-induced changes in growth hormone levels, calcitonin, parathyroid hormone, and vitamin D do not explain the observed bone loss. Recent findings, however, suggest that the observed changes in osteoblast growth are caused by alterations in the basic molecular mechanisms affected by microgravity [2]. The elucidation of the factors which affect the modulation of bone cell growth will allow a direct approach to evaluating and counteracting the debilitating effects of microgravity on bone formation.

There is mounting evidence that PGE_2 increases bone cell growth and plays a role in normal bone formation. *In vitro* stimulation of cultured bone cells with growth factors enhances endogenous prostaglandin production [3–6]. In space flight, crew members have a significantly reduced mechanical load which is thought to play a role in bone loss. Direct mechanical stimulation of cat bone tissue has been shown to increase the synthesis of PGE_2 *in vivo* [7]. Jee *et al.* [8] have demonstrated that PGE_2 stimulates trabecular bone formation *in vivo* by 120% over a 21-day period. Prostaglandins of the E series are now described as autocrine and paracrine factors in osteoblast and osteoclast activity and remodeling. In addition, PGE_2 stimulates osteoblast replication and new bone formation, but to date no evidence has been found to explain its effect on bone growth. Osteoblasts that are mechanically stressed in culture synthesize and release the PGE_2. This laboratory has recently reported that osteoblast growth activation by serum is accompanied by an increase in PGE_2 synthesis. Activation of osteoblast

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0014-4827/96 $18.00

growth is significantly decreased by inhibition of endogenous prostaglandin synthesis, and addition of exogenous prostaglandins results in significant partial restoration of cell growth [9]. More important is our recent observation that PGE_2 stimulates expression of the early growth regulator genes *c-fos* and *c-jun* [10]. In these studies we show a reduction in serum growth activation in microgravity. We also found that osteoblast growth activation with sera in microgravity fails to stimulate prostaglandin synthesis. These data suggest that microgravity may play a role in osteoblast serum growth activation at the cellular level.

METHODS

Preparation of osteoblasts. The osteoblast cell line was kindly provided to us by Dr. M. Kumegawa (Josai Dental University, Japan) and cells were grown as previously described [11] MC3T3-E1 cells were grown in alpha minimal essential medium (aMEM) (Sigma Chemical Co., St. Louis, MO) with 10% fetal calf serum (FCS) (Hyclone Labs Inc., Logan UT), containing antibiotics (100 U penicillin G/ml, 0.01 mg streptomycin/ml, 0.25 mg amphotericin B/ml, 0.0091 NaCl) and 2 m*M* L-glutamine (Sigma). At the time of preparation for flight, the cells were trypsinized and seeded on coverslips that were placed in the Materials Dispersion Apparatus (MDA) FCS-medium. Cells were activated during space flight in the MDA with addition of 10% FCS-media.

Cell morphology. Upon return, cell nuclei were stained with Hoechst 33258 (Sigma) as previously described [12] and F-actin was stained with rhodamine phalloidin (Molecular Probes, Eugene OR). Cell growth was quantified using microscopic counting of cell nuclei on coverslips by counting multiple slides for a total of five fields chosen at random at the center and at the edges of the slides. A portion of the photographs were scanned and size analyzed using NIH image 1.58 software.

Description of the MDA Minilab hardware. Hardware for these experiments consisted of the Materials Dispersion Apparatus (MDA) Minilabs, developed by Instrumentation Technology Associates (ITA), of Exton, PA. These brick-sized units consist of two sliding blocks with sample wells in opposite faces. The blocks, misaligned during launch, are aligned in microgravity to bring fluids in the wells of top and bottom blocks into contact. Each of the test wells in the bottom block were modified to accommodate two small coverslips preseeded with osteoblasts.

Experiment details. The experiment was launched on STS-56 on April 8, 1993 and recovered on April 17 after a nominal 9-day mission. For the preiaunch and orbit insertion phases, cells were maintained at ambient temperature (20 to 23°C) in medium containing 0.5% FCS to retard growth. Prior to activating the osteoblasts in microgravity, the temperature was increased to 37°C and blocks were moved to bring the first top well into alignment with cells. The top well contained culture medium with a concentration of FCS required to bring the final concentration in the top/bottom well combination to 10%. This served to activate cell growth. After 4 days in microgravity, the blocks were moved again to bring the second top well, containing formalin calculated to give 3% final concentration, into contact with the cells to fix them while still in microgravity. The temperature was then set at 20°C for the remainder of the mission. After landing, the experiment was transported to Hangar L at the Kennedy Space Center where the coverslips were removed from the MDA minilabs and shipped to the Principal Investigators' laboratories for analysis. A concurrent ground control experiment in 1-gravity (1-G) in the same type of hardware was conducted for the comparison to flight.

Glucose analysis. Culture medium samples were evaluated for glucose content with a Beckman glucose analysis reagents kit (Beckman, Brea, CA) using a Beckman Model 2 glucose analyzer (Beckman).

Measurement of PGE_2. A competitive enzyme immunoassay (EIA, Cayman Chemical, Ann Arbor, MI) was used to assess the PGE_2 levels in the osteoblast media. Microtiter 96-well plates were purchased already precoated with a mouse monoclonal antibody that binds the Fc site of rabbit IgG. The addition of given quantities of PGE_2 standard or dilutions of unknown samples to appropriate wells was followed by the addition of the tracer (PGE_2 covalently bound to electric eel acetylcholinesterase). This tracer did not show altered binding affinity to the rabbit anti-PGE_2 antibody. Finally, a given quantity of rabbit anti-PGE_2 antiserum was added which allowed the tracer and standard or unknown amounts of PGE_2 in the wells to compete for binding. The anti-PGE_2 antibodies in turn were bound to the mouse anti-IgG coating the bottom of the wells. Following an overnight incubation, the unbound reagents were rinsed from the wells and a specific acetylcholinesterase substrate, Ellman's reagent, which contains the substrate acetylcholine and 5,5'-dithio-bis-2-nitrobenzoic acid was added to each well. During the development time, metabolized acetylcholine reacted with the thiol groups of the 5,5'-dithio-bis-2-nitrobenzoic acid, creating a chromophore that absorbs at 412 nm. The intensity of the absorbance was directly proportional to the amount of bound tracer and therefore inversely proportional to the amount of nontracer PGE_2 present in the standards or samples. All standards or samples were run in duplicate. The anti-PGE_2 antibody has a 9.3% cross reactivity with 15-keto-PGE_2, 5% with PGE_1 and <0.1% with all the other major prostaglandins and thromboxanes.

RESULTS

Reduction in cell growth activation in microgravity. The flight and ground cells were plated and grown for

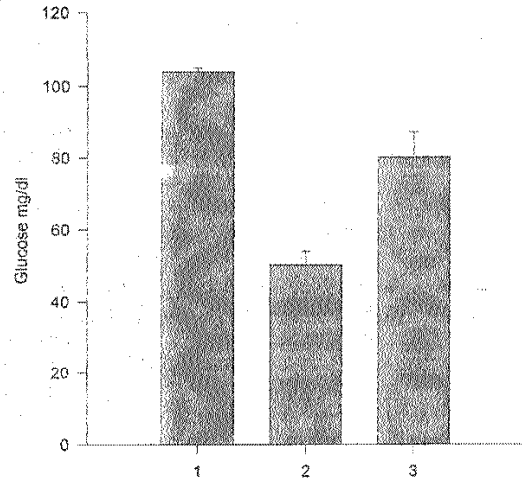

FIG. 1. Comparison of glucose remaining in media of flown (3) and ground (2) cells after 4 days in microgravity. Cells were maintained at 20°C on medium containing 0.5% fetal calf serum for approximately 30 h prelaunch plus 12.45 h in microgravity. The temperature was then increased to 37°C and the cells were stimulated by addition of an equal volume of medium containing 10% fetal calf serum. Glucose levels represented are (1) media control after 4 days; (2) glucose levels in ground control osteoblasts after 4 days; (3) glucose levels in flight osteoblasts after 4 days. $n = 4$ chambers (MDA) or samples (ground media). Error bars represent ± SD.

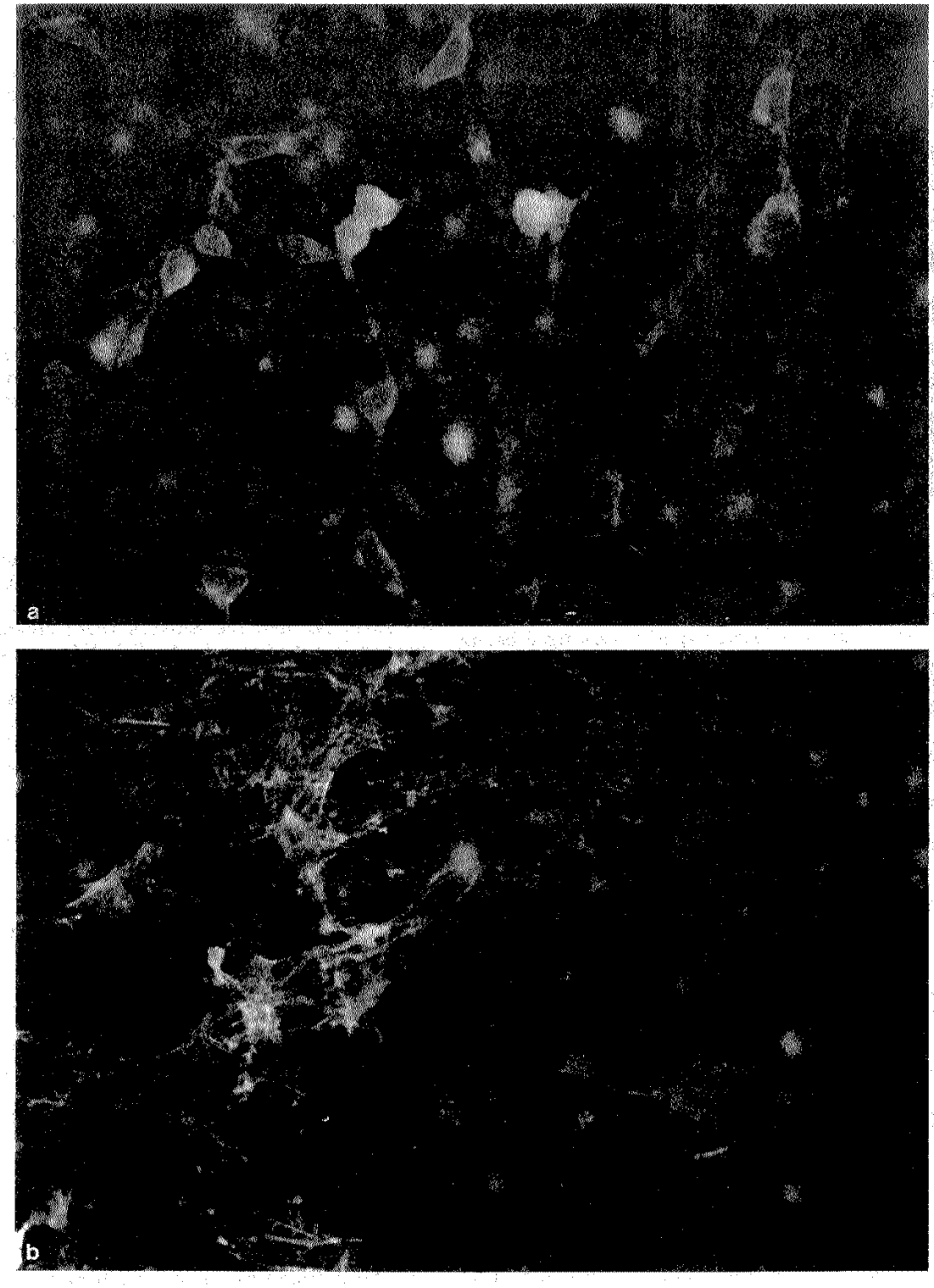

FIG. 2. Structure of the actin cytoskeleton of flown (a) and ground control cells (b) using a Zeiss Neofluar 100× oil objective. The F-actin in osteoblasts grown on the coverslips was stained with rhodamine-phalloidin and the actin cytoskeleton was visualized by fluorescence microscopy. (a) Flight samples, (b) ground controls.

TABLE 1

Reduced Growth of Osteoblasts in Microgravity

Condition	Number of nuclei in field	Relative average size (total pixels in nuclei)
Ground (1-G)	141 ± 8	42 ± 6.5
Flight (microgravity)	55 ± 6*	29 ± 6*

Note. Osteoblast cells were plated in 2% FCS-media on glass coverslips before being placed in MDA hardware. Prior to loading, coverslips were dipped in medium without serum to remove excess FCS. Duplicate sets of coverslips were placed in quadruplicate wells in ground and flight hardware in 0.5% FCS media prior to hand-off to shuttle. Cells were held in a quiescent state over the next 30 h prior to launch. At 16 h after launch, the flight (microgravity) and ground (1-G) cells were activated with 10% FCS-media and allowed to grow for 4 days before fixation. Upon return to earth, the cell nuclei were stained and counted. Cells were counted under 100× objectives and cell size was determined by relative total pixel area by scanning the field using the NIH 1.58 image software program. Results are reported as the mean ± standard deviation.

$P < 0.0001$ ($n = 4$ fields for each condition).

16 h before flight. Under these conditions, cells were quiescent upon entry into microgravity. The cells were activated with 10% serum after 16 h of microgravity exposure. Four days later, the cells were fixed before reentry and landing.

Glucose utilization. The medium removed from the cell cultures after landing was analyzed for glucose content in both the flight and 1-G samples. The presence of fixative in the medium did not affect the glucose assay (data not shown). As seen in Fig. 1 the flown cell utilized 50% less glucose than the 1-G controls ($P < 0.001$). When glucose utilization is calculated on a per-cell basis, ground and flight samples had comparable values.

Cell cytoskeleton. The fixed cells were stained with rhodamine phalloidin to visualize the F-actin fibers. As seen in Fig. 2b, the 1-G osteoblasts have a typical morphology with well-attached cells and multiple actin stress fibers as is characteristic of the MC3T3-E1 osteoblast. In Fig. 2a, the flown osteoblasts exhibit a distinctly different morphology with reduced stress fibers and elongated extended podia and rounded centers around the nucleus.

Nuclei. Despite the same experimental treatment, the Hoescht-stained nuclei had a distinctly different morphology and staining pattern in the 1-G controls compared to the flown osteoblasts. As seen in Table 1, the cell number was significantly lower in the flown osteoblasts when compared to the 1-G controls. As seen in Fig. 3b, the control nuclei have a punctate staining pattern and a rounded appearance with a significantly larger nuclear size compared to the flown cells in Fig. 3a, which have an oblong shape and reduced punctate staining. In addition to changes in morphology, there was significant reduction in cell growth and nuclei size. As seen in Table 1, the growth of cells was significantly ($P < 0.0001$) inhibited in space. The average size of nuclei is also reduced in the flown cells as compared to ground controls ($P < 0.0001$), as shown in Table 1.

No serum activation of prostaglandin synthesis in microgravity. After 16 h of microgravity exposure, the flown cells had a significant increase in prostaglandin synthesis ($P < 0.009$). The steady state prostaglandin E_2 content in the medium was significantly increased after 4 days by the endogenous synthesis of the 1-G osteoblasts after growth activation. There was no increase in the steady state prostaglandin E_2 content of the flown cells 4 days following serum treatment (Fig. 4). However, on a per-cell basis, at 4 d, prostaglandin synthesis was comparable for flight and ground samples.

DISCUSSION

In microgravity astronauts have a reduction of mechanical stress on the skeletal system. Although in-flight exercise is a helpful countermeasure used by both the American and the Russian space programs, the greatest losses of bone in the U.S. Flight program 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 percent of bone over the 84-day mission period [13]. However, in the 237-day Soviet Soyuz T-10 mission, the Cosmonauts lost from 13–19% of bone in spite of 2–4 h 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 to 19.8% over an 84- to 184-day period [14, 15]. The loss of bone in the presence of consistent exercise suggests that additional molecular mechanisms are responsible for bone loss.

PGE_2 is known to be released by bones *in vivo* [7, 16] and *in vitro* [17] as a result of mechanical stress. The addition of exogenous prostaglandin increases osteoblast growth and can overcome a dexamethasone inhibition of bone growth (dexamethasone is a glucocorticoid which inhibits cytosolic phospholipase 2 which reduces prostaglandin synthesis). Space flight crews experience both decreased mechanical stress and increased levels of cortisol (a naturally occurring glucocorticoid), which could directly affect crewmen prostaglandin synthesis. The resulting down-regulation of prostaglandin synthesis (Fig. 4) could potentially explain the bone loss observed in astronauts.

Evidence from this and other laboratories points to PGE_2 as a local bone growth regulator. Jee *et al.* [8] have demonstrated that PGE_2 stimulates trabecular bone formation *in vivo* by 120% over a 21-day period.

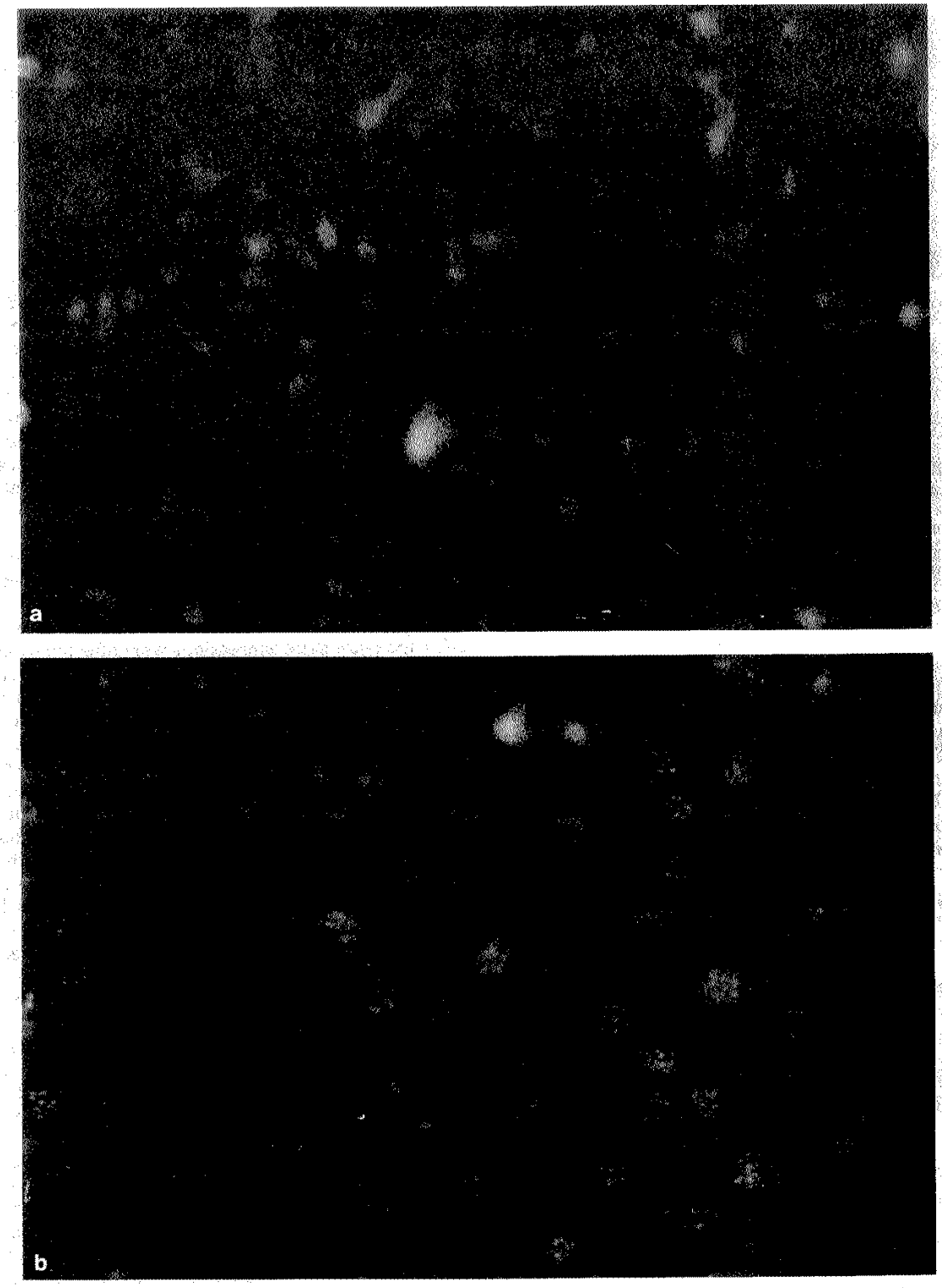

FIG. 3. Nuclear morphology of flown (a) and ground control (b) cells using a Zeiss Neofluar 100× oil objective. Cells on coverslips in the presence of 10% fetal calf serum containing medium for 4 days were stained after the mission with Hoechst 33258. Nuclear morphology is shown in the fluorescence micrographs for (a) flown and corresponding (b) ground control cells.

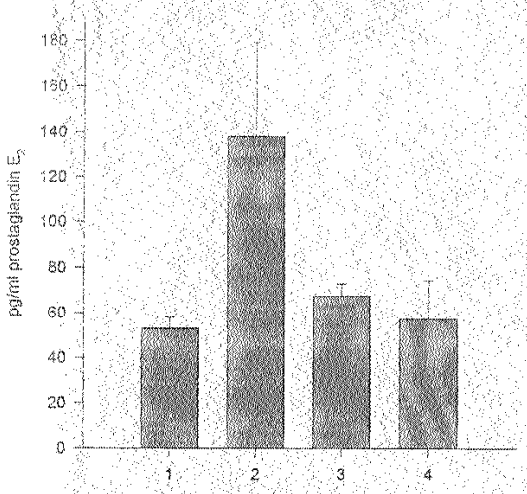

FIG. 4. Prostaglandin synthesis in culture medium from flown and ground control cells. The cells were treated as described in the legend to Fig. 1 and prostaglandin synthesis was measured in the culture medium as described under Materials and Methods. Prostaglandin levels in (1) ground control after 16 h; (2) prostaglandin levels in ground control after 4 days; (3) prostaglandin levels in flight after 16 h; (4) prostaglandin levels in flight after 4 days are shown in this figure.

Animals treated with PGE have been noted to have increased proliferation of their long bones [18]. Moreover, infants with cyanotic congenital heart disease treated with prostaglandins to close the ductus have been observed to have cortical proliferation of long bones [19, 20]. In contrast to parathyroid hormone and calcitonin, prostaglandins have also been shown to cause an increase in the incorporation of proline into collagen and noncollagen proteins in chick bone [21]. Prostaglandins are produced by osteoblasts and are likely acting as local bone regulating hormones. *In vitro* growth factor stimulation of cultured bone cells enhances prostaglandin production [3–6]. Prostaglandins of the E series are autocrine and paracrine factors in osteoclast activity and remodeling [23]. In addition, PGE_2 stimulates osteoblast replication and new bone formation, but to date no evidence has been found to explain its effect on bone growth. The evidence from space flight experiments [13] strongly supports the idea that microgravity-induced bone loss is due to an inhibition of bone formation and not to alterations in the normal bone breakdown mechanisms.

In this study, we found that the flown osteoblast cells were in good condition postflight; however, they had a significantly changed morphology when compared to the 1-G controls. Actin cytoskeleton demonstrated distinct changes in both morphology and stress fiber content when the cells were activated in microgravity (Figs. 2a, 2b). There is evidence of inhibited serum growth stimulation in microgravity compared to the ground-based control. This is demonstrated by reduced glucose utilization as a result of reduced cell number.

The reason for this reduced growth activation by sera in microgravity is not known; however, the work of deLaat's group has partially described the relationship between gene expression and microgravity [24–28] by demonstrating that a number of responses of A431 cells to epidermal growth factor (EGF) are affected by microgravity. The responses include a decrease of *c-fos* and *c-jun* induction and serum element activity [24]. In sounding rocket experiments (where cells experience microgravity for 6 min), they demonstrated that sera and or EGF-induced expression of the proto-oncogenes *c-fos* and *c-jun* were reduced almost fourfold in microgravity. The decreased gene inductions were attributed to possible alterations in the response of the *c-fos* promoter–enhancer regions. The nuclear proto-oncogenes *c-jun* and *c-fos* are known regulators of DNA synthesis [25] and are required for entry into S-phase DNA synthesis [26].

In later studies, deLaat's group demonstrated that the nuclear responses to protein kinase C signal transduction were sensitive to gravity changes [27]. These studies demonstrate that phorbol ester (TPA)-induced *c-fos* and *c-jun* gene expressions were affected by microgravity, while there were no changes in the calcium response, thus implicating the diacylglyceride portion of the PKC signal transduction. In later work, they demonstrated that the 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. This suggests that microgravity influences EGF-induced signal transduction not at the EGF binding and receptor redistribution level, but upstream of early–immediate gene expression [28]. Finally, deLatt has demonstrated that EGF-induced actin cytoskeleton changes are affected by the arachidonic acid pathways through cyclo-oxygenase (prostaglandin) and 5-lipoxogenase (leukotriene).

The requirement of prostaglandin synthesis for activation of osteoblast growth was demonstrated when we blocked growth and prostaglandin synthesis with dexamethasone, and reestablished growth by addition of exogenous PGE_2 [9]. Our recent finding of PGE_2 specific induction of *c-fos* mRNA expression and growth activation in the osteoblast suggests that the osteoblast growth activation by PGE_2 may work through a *c-fos* mediated growth stimulation. It is possible that the reduction in serum-induced osteoblast growth and prostaglandin synthesis in microgravity may share a similar reduction of the early–immediate gene expression as described by deLaat's group. The mechanism which causes the downregulation of growth activation is not known; however, studies from others have shown changes in membrane structure and rearrangement of

cytoskeletal elements, either of which could alter sera mediated gene activation of receptors, cytoskeletal response, and cell signaling mechanisms.

It is unknown if the bone loss seen in astronauts during flight is due directly to the lack of mechanical stress (1-G force) in microgravity, systemic changes in hormones, alterations in the signal transduction by serum growth factors, or to changes in signal transduction at the molecular level. In this paper, we have shown that in microgravity quiescent osteoblast cells exhibit reduced serum growth activation which results in reduced prostaglandin synthesis, and reduced glucose utilization, suggesting that at least a portion of the reduced bone formation seen in space flight may be due to a direct effect of microgravity on osteoblast growth regulation.

This work was supported by NASA Grants NAGW-1244 (MHF), NAG-2-981 (MHF), and NAG 2-1009 (M.L.). This work was also supported by the Department of Veterans Affairs Secretary's Special Award (M.H.F.). We thank Nona Baker for editorial assistance and Don Lum, Vicki Vincent, and Ray Tjandrawinata for their thoughtful comments.

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Received June 8, 1995
Revised version received December 28, 1995