Osteoblast fibronectin mRNA, protein synthesis, and matrix are unchanged after exposure to microgravity

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ABSTRACT **The well-defined osteoblast line, MC3T3-E1 was used to examine fibronectin (FN) mRNA levels, protein synthesis, and extracellular FN matrix accumulation after growth activation in spaceflight. These osteoblasts produce FN extracellular matrix (ECM) known to regulate adhesion, differentiation, and function in adherent cells. Changes in bone ECM and osteoblast cell shape occur in spaceflight. To determine whether altered FN matrix is a factor in causing these changes in spaceflight, quiescent osteoblasts were launched into microgravity and were then sera activated with and without a 1-gravity field. Synthesis of FN mRNA, protein, and matrix were measured after activation in microgravity. FN mRNA synthesis is significantly reduced in microgravity (0-*G*) when compared to ground (GR) osteoblasts flown in a centrifuge simulating earth's gravity (1-*G*) field 2.5 h after activation. However, 27.5 h after activation there were no significant differences in mRNA synthesis. A small but significant reduction of FN protein was found in the 0-*G* samples 2.5 h after activation. Total FN protein 27.5 h after activation showed no significant difference between any of the gravity conditions, however, there was a fourfold increase in absolute amount of protein synthesized during the incubation. Using immunofluorescence, we found no significant differences in the amount or in the orientation of the FN matrix after 27.5 h in microgravity. These results demonstrate that FN is made by sera-activated osteoblasts even during exposure to microgravity. These data also suggest that after a total period of 43 h of spaceflight FN transcription, translation, or altered matrix assembly is not responsible for the altered cell shape or altered matrix formation of osteoblasts.—Hughes-Fulford, M., Gilbertson, V. Osteoblast fibronectin mRNA, protein synthesis, and matrix are unchanged after exposure to microgravity. *FASEB J.* 13 (Suppl.), S121–S127 (1999)**

Key Words: growth activation · spaceflight · mRNA expression

THE UNIQUE ENVIRONMENT of spaceflight places unusual stress on, and causes many physiological changes in, organisms that evolved in a 1-*G* environment (1–3). Some of the basic physiological changes include muscle atrophy and loss of calcium and mineralized bone. The bone loss that accompanies spaceflight is one of the most serious health hazards associated with, and impediments to, long-term manned missions (4, 5). Biomedical studies of manned spaceflight have consistently indicated a continuous and progressive loss of calcium and weight-bearing skeletal bone. Evidence from animal studies suggests that the bone loss occurring in spaceflight is due to a decrease in bone formation (6). The decrease in bone formation and osteoblast growth in microgravity is likely due to both indirect effects such as systemic or hormonal changes in the body and direct effects such as cellular response to the lack of mechanical stress.

Proper cell attachment to the extracellular matrix (ECM)[2] is required for normal cell growth (7). Previous studies have demonstrated that bone exhibits altered ECM assembly of collagen (8–9), actin (10), and altered cell morphology and nuclear shape of osteoblasts (9–12) in microgravity. The cell surface adhesion protein, fibronectin (FN), mediates the attachment of a variety of cell types to other ECM components such as type I collagen, heparin, and proteoglycans. Cell binding to FN is mediated by integrins, which recognize the arginine-glycine-aspartic acid (RGD) sequence on the FN molecule. The integrins on the cytoplasmic surface bind talin and α-actinin, forming a link between ECM components and the cytoskeleton. Binding of FN can also be mediated by RGD-independent interactions such as $\alpha_4\beta_1$ and proteoglycan receptors including CD44 and syndecans (13). FN is known to be a factor in cell shape and growth regulation (14, 15). It has been demonstrated that FN adhesion, resulting in the

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[2] Abbreviations: FN, fibronectin; ECM, extracellular matrix; RGD, arginine-glycine-aspartic acid; GR, ground; α-MEM, α-minimal essential medium; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

ability of epithelial cells to spread over a planar surface, can promote cell cycle progression from G_0 to S phase (16). Although the mechanisms are still under investigation, FN has also been shown to increase mechanochemical signal transduction intermediates in fibroblasts (17). FN is present during all stages of matrix-mediated bone formation with synthesis rates being high during cell proliferation and attachment (18). In addition, blocking FN attachments has been shown to inhibit osteoblast differentiation and in mature bone cultures resulted in apoptosis (19).

For these reasons we were interested in determining what effect microgravity would have on the FN message, protein, and matrix organization. To define the role of FN in the altered cell shape and altered ECM matrix formation we performed space shuttle experiments in Biorack hardware in the following gravity environments: ground (GR), 0-*G* flight (0-*G*), and 1-*G* flight (1-*G*) conditions. Early passage MC3T3-E1 osteoblasts were used because they have been shown to exhibit osteoblast behavior during both proliferation and differentiation stages of bone mineralization (20, 21). We have demonstrated previously that these osteoblasts change cell shape in microgravity and that osteoblast genes are induced by application of gravity and vibration forces (22–24). In this study, we asked whether FN plays an important role in microgravity-induced changes in cell shape and matrix formation. Therefore, analysis of FN gene induction, protein synthesis, and matrix formation was done on samples collected under different gravity conditions during spaceflight. Our results demonstrate that extended exposure (>24 h) to microgravity does not cause changes in FN synthesis and matrix.

MATERIALS AND METHODS

Cell culture

The MC3T3-E1 osteoblast cell line is clonally derived from embryonic mouse calvaria (17, 18) and was kindly provided to us by Dr. M. Kumegawa (Josai Dental University, Japan). The cell line was maintained at low passage. Cells were grown in alpha minimal essential medium (α-MEM) with 10% fetal calf serum (Hyclone Labs Inc., Logan, UT) supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO), 25 mM HEPES, and antibiotic-antimycotic solution (100 U penicillin/ml, 0.01 mg streptomycin/ml, 0.25 mg Amphotericin B/ml). Cells were grown in a 37°C incubator with 5% CO_2. They were fed three times a week and passed when the cells reached confluence. For flight experimental samples, 120,000–200,000 cells were plated onto non-coated, sterile 11 × 22-mm glass coverslips (Thomas Scientific, Swedesboro, NJ) placed in six-well plates, and grown in 10% serum containing α-MEM overnight. Cell-coated coverslips were transferred into the plungerbox units in 2% serum-containing medium for flight. In cooperation with NASA flight rules, the plungerbox units were held for 17 h in the shuttle at mid-deck temperature before launch. This, combined with low serum-containing media placed the osteoblast in a quiescent state before space shuttle launch. Many previous culture experiments sent into space have been actively growing at the time of launch. Moreover, lack of refrigeration has previously limited on-board sample preservation and therefore many experiments were terminated after landing on earth. Even for the few experiments that had the opportunity for on-board collection, the data were compromised by flight-imposed limitations including the following: lack of sufficient sample numbers, addition of supplements directly affecting gene expression, lack of fresh media changes, and lack of on-board 1-*G* controls. To achieve our goal of studying FN metabolism 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- and 1-*G* flight samples and ground samples. Refrigerator and freezer space was available for reliable sample storage after collection in microgravity. In contrast to some previous spaceflight experiments, media supplements such as dexamethasone, β-glycerol phosphate, and ascorbic acid were not added to our media because these agents are known to directly affect gene expression and cell morphology of the osteoblast.

Biorack facility and osteo hardware

Biorack is a multi-user facility that consists of incubators with variable gravity centrifuges, a cooler, a freezer, and a sealed glovebox. Two identical Biorack modules were used: one remained on earth and the other was integrated into SpaceHab and flown on the space shuttle. Biorack has an important advantage over other microgravity facilities in that it provides a small-radius (78 mm) slow-rotating (107.0 ± 0.5 rpm) centrifuge. The centrifugal force results in a 1-*G* on-board control. Because of proximity, both the 0-*G* and 1-*G* samples experience identical launch vibrations, accelerations, cosmic radiation, and other unknown conditions of flight. The only difference between the 0-*G* and 1-*G* flight groups is the gravity parameter. In addition, an identical experiment was performed in the Biorack module on earth with a 2-h delay from in-flight procedures.

The "Osteo" experimental flight hardware was designed according to European Space Agency specifications for use in the Biorack facility and constructed by Centrum voor Constructie Mechatronica (CCM, Neuenen, The Netherlands). The hardware consisted of the CCM plunger box and its Type I container developed for spaceflight 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 independent culture chambers that each hold two 11 × 22-mm glass coverslips. Each culture chamber was a separate sample. For each condition a sample size of $n = 4$ was used. Each culture chamber has series of compartments filled by either 10% serum-containing α-MEM or fixative, which can be exchanged into and out of the cell culture compartment by manually releasing a spring-loaded plunger.

Experimental time line

Quiescent cells were stored in the mid-deck locker until 18 h after launch. The astronauts then transferred the samples into a 37°C incubator (either in the 1-*g* centrifuge or 0-*g* static rack) for 1 h before stimulating the cells to grow by changing their media from 2 to 10% serum-containing medium ($t = 0$). Zero-*G* and 1-*G* samples were fixed during flight with a

modified guanidinium thiocyanate solution (for RNA preservation) $t = 0$, 2.5 h, and 27.5 h or 3.7% formaldehyde solution (for morphology) at 27.5 h. Samples fixed with the guanidinium thiocyanate solution or the formaldehyde solution were stored at −20°C or +5°C, respectively, for the remainder of the space shuttle mission. There were four separate samples for each gravity condition at each time point.

RNA isolation, reverse transcription (RT), and polymerase chain reaction (PCR)

RNA from cultured MC3T3-E1 osteoblast cells was extracted by a modified guanidinium thiocyanate method, which was based on the protocol previously described by Chomczynski and Sacchi (25). Linear RT-PCR was performed as previously described (9, 19, 20, 23–26). Because sample size was so small (200,000 cells) RNA content was held constant and linear RT-PCR was accomplished by varying the number of PCR cycles. PCR conditions were established so that the amplification reaction was stopped in the linear range and reaction products could be accurately quantified and compared. Oligonucleotide primers were designed to span at least one intron in order to detect any contaminating genomic DNA. FN primer sequences were designed from Genebank sequences by MHF: sense, 5′-CCC CAG TGA TGT TAG CAG ACC; anti-sense, 5′-GAT GGC AAA AGA AAG CAG AGG. Operon Technologies, Inc. (Alameda, CA) synthesized the oligonucleotides. PCR bands were identified by agarose gel electrophoresis and photographed with a direct screen instant camera DS-34 (Polaroid Corp., Cambridge, MA). For quantification, the bands were scanned at 400 dpi with a LaCie Silver Scanner III (LaCie, Portland, OR) and stored as TIFF files. The area and density of each band were determined using Sigma Gel (SPSS, Inc., Chicago, IL). The total number of pixels was corrected to the micrograms of RNA used for each RT-PCR. Sample size for all conditions was $n = 4$. Descriptive statistics and unpaired Student's t test analysis was performed with Sigma Stat v. 2.0 (SPSS, Inc.).

Protein isolation

Protein remaining in the guanidinium thiocyanate solution after the RNA extraction was precipitated out of each sample using a method similar to that previously described (27). Briefly, 2.5 ml of isopropanol was mixed with the remaining organic phase from the RNA extraction, incubated at room temperature for 15 min, and centrifuged at 12,000 g for 10 min at 4°C. The resulting protein pellet was washed three times in 4 ml of 0.3 M guanidine hydrochloride in 95% ethanol. For each wash samples were incubated in the wash solution at room temperature for 20 min followed by centrifugation. Protein was resuspended in 2% sodium dodecyl sulfate (SDS). Total protein was quantified for each sample in triplicate by DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol and read on a Dynatech MR5000 96-well plate reader. The standard curve and protein quantification results were interpreted with BioLinx 2.0 software (Dynatech Laboratories, Chantilly, VA).

Western blots

Five micrograms of total protein for each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% polyacrylamide). Two molecular weight markers were also loaded onto the gel: a prestained molecular weight marker (Bio-Rad) was used to track the migration of the samples through the gel and a Cruz Marker™ (Santa Cruz Biotechnology, Santa Cruz, CA), which is compatible with the horseradish peroxidase-conjugated secondary antibody and can be visualized by chemiluminescence, and was recorded directly onto the film. Proteins were transferred onto nitrocellulose filters with 100 volts for 1 h. No detectable protein, as measured by staining, remained in the gel after transfer. Filters were blocked with 5% blotto (20 mM Tris, 150 mM NaCl, pH 7.6, with 5% nonfat milk and 0.02% Tween-20) for 2 h. Primary antibodies for FN were purchased from DAKO (Carpinteria, CA). Molecular weight markers and secondary antibodies were purchased from Santa Cruz Biotechnology. Proteins were visualized by enhanced chemiluminescence (Amersham Life Sciences Inc., Arlington Heights, IL) and recorded on Kodak XAR-5 film. To ensure that the Western blot signal was within a linear range, the amount of protein loaded on the gel, primary and secondary antibody concentrations, and film exposure length were all optimized. Films were digitized with an Epson Expression 836XL scanner into Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). Density analysis was performed with the Sigma Gel program (SPSS, Inc.). All densitometry readings were normalized to nanograms of total protein loaded onto the gel. Sample size for each condition was $n = 4$. Statistical analysis was performed in Sigma Stat and graphed with Sigma Plot (SPSS, Inc.).

Cell morphology

Osteoblasts were fixed on coverslips with 3.7% formaldehyde in phosphate-buffered saline (PBS) during flight and stored at 5°C for 1–2 weeks before staining. There was no visible difference between control samples that were immediately stained and samples stored in formaldehyde at 5°C. For FN visualization, coverslips were blocked with 10% goat serum in PBS, incubated with anti-FN (rabbit) primary antibody (DAKO), and then incubated with anti-rabbit secondary labeled with Texas Red. Dried osteoblast coverslips were mounted onto slides and photographed with a Zeiss Axioscope microscope at ×40 and ×100 magnification. Slides were processed at the same time under identical conditions. Photographs were taken at identical exposure times and conditions. Photographs were taken at predetermined areas of the samples. Slides were digitized with an Epson Expression 836XL scanner into Adobe Photoshop for computer analysis with NIH image. Controls not shown were incubated with protein blocker, in the absence of FN antibody, and then with the secondary antibody for the appropriate incubation times.

RESULTS

FN matrix

To compare the organization of the FN portion of the ECM under different gravity conditions, osteoblast cells were launched on the space shuttle in a semi-quiescent state and were sera activated 19 h after reaching orbit. Twenty-seven and one-half hours after activation, the osteoblasts, which were grown on untreated glass coverslips in microgravity (0-G) or in a 1-G centrifuge on board the space shuttle (1-G) or ground controls (GR), were fixed with formaldehyde for visualization of the FN matrix on conclusion of the flight.

Samples were treated with a polyclonal antibody

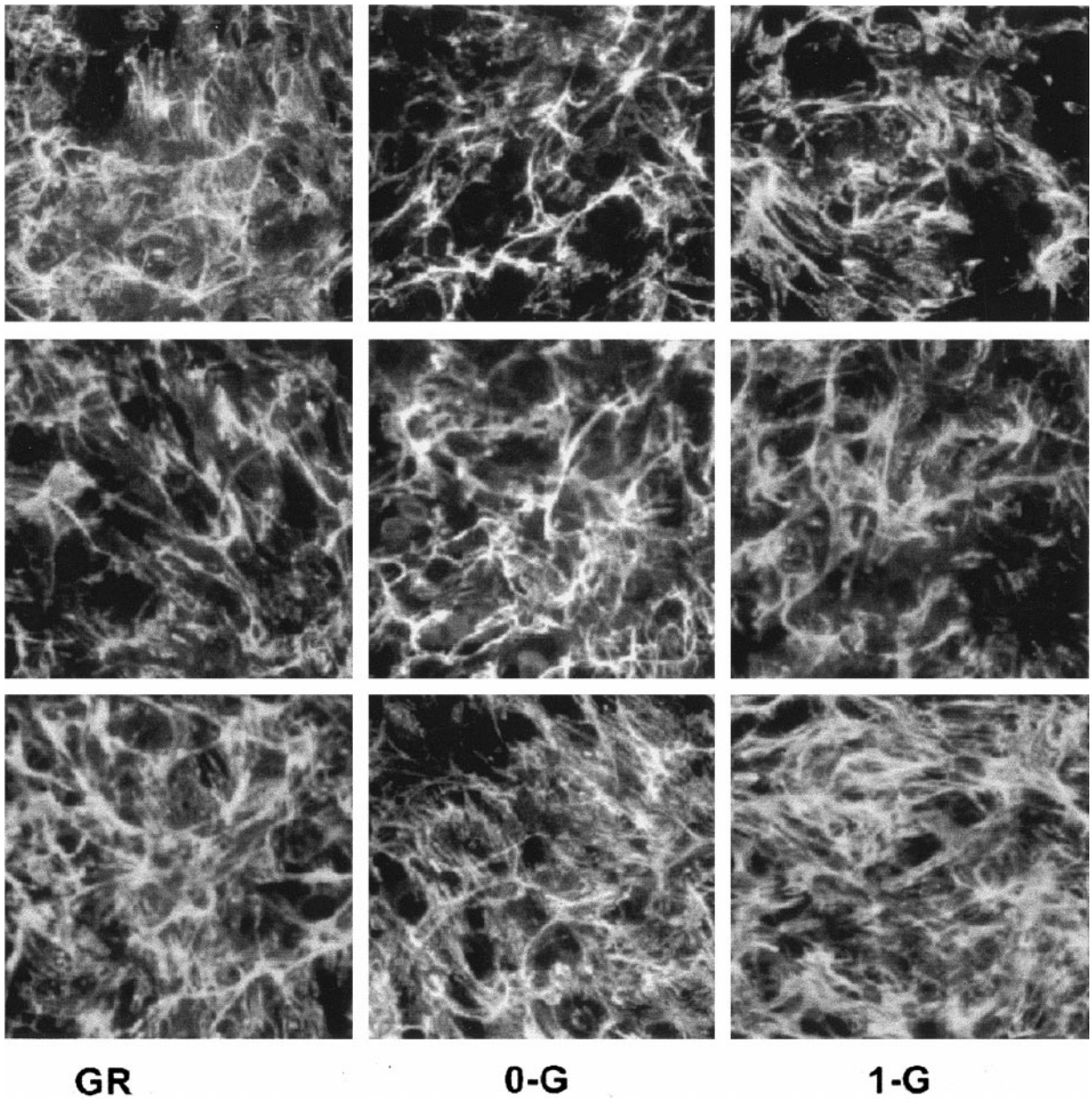

Figure 1. Immunofluorescence localization of FN in MC3T3-E1 osteoblast cultures grown on earth (GR), in microgravity (0 *G*), or in an on-board 1-*g* centrifuge (1 *G*). Cells were seeded on non-treated glass coverslips and grown in 10% fetal calf serum overnight. Cells were sera deprived before launch and sera activated in microgravity 19 h after launch. Twenty-seven and one-half hours after activation, while still in microgravity, cells were fixed with formaldehyde. Cells were stained with an anti-FN primary antibody and then with anti-rabbit secondary antibody labeled with Texas Red. Photographic exposure times and conditions were identical using a ×100 objective.

that selectively recognizes cellular FN to immunolocalize the FN matrix. The majority of the FN matrix had a diffuse distribution throughout the ECM. After approximately 1 day in microgravity there were no apparent changes in the FN matrix orientation between the 0-*G* and the 1-*G* or GR samples (**Fig. 1**). Quantification of total fluorescence from randomly selected slides revealed no significant differences among the different gravity conditions (**Table 1**). As an additional control, in samples without primary antibody, there was no FN matrix seen (image not shown).

FN protein synthesis

The Western blot shown in **Figure 2** shows representative samples of the relative amount of FN per cell

TABLE 1. *Quantification of Total Fluorescence*

Condition	Total density (pixels)
Ground	1,676 ± 988
0 *G*	1,231 ± 829
1 *G*	1,415 ± 1193

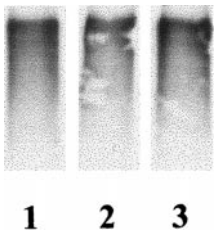

Figure 2. Representative data from FN Western blot analysis. Osteoblast cultures were sera activated in microgravity. Total protein extracts were obtained from osteoblast grown at GR (lane 1), 0-*G* (lane 2), and 1 *G* (lane 3). Five micrograms of total protein was loaded per lane. Sample size was $n = 4$ for each condition.

because proteins were extracted from samples plated with an equal number of cells. Most of the immunoreactive material migrated to the position of authentic FN, although some lower molecular mass was also seen. In control blots performed without primary antibody present, these bands were not seen (data not shown). At the 2.5 h timepoint there was a small but significant reduction of FN protein in the 0-*G* sample when compared to GR samples (**Fig. 3**). This difference was lost at the later timepoint, where there was no significant difference in total FN protein between the various gravity conditions. FN protein content is evident 27.5 h after activation. FN protein is shown to have increased by approximately fourfold during the 25 h between the two time points.

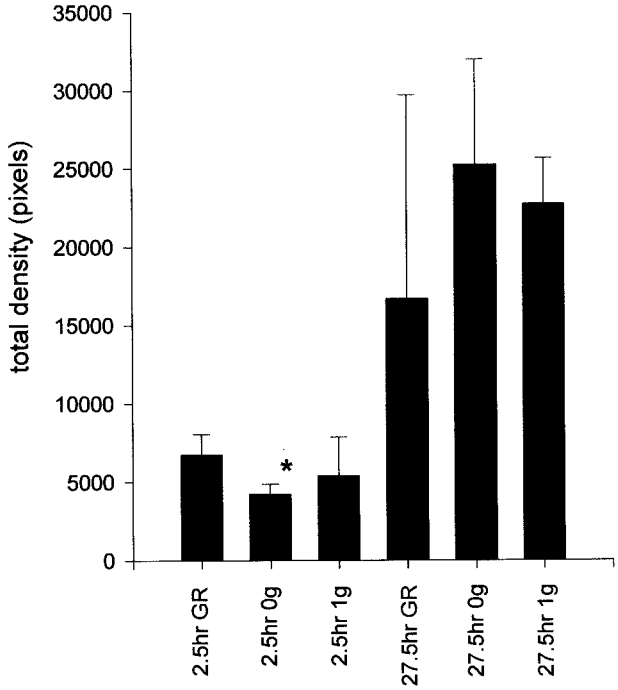

Figure 3. FN protein quantification. Western blot films from 2.5 h and 27.5 h samples were digitized and quantified as described in Materials and Methods. For each condition the samples size was $n = 4$ and presented as total pixels ± SD. In the 2.5 h samples there was a significant difference between the 0 *G* and GR samples ($P < 0.030$). There was approximately a fourfold increase in FN protein in the 27.5-h samples compared to the 2.5 h samples with no significant difference between the gravity conditions.

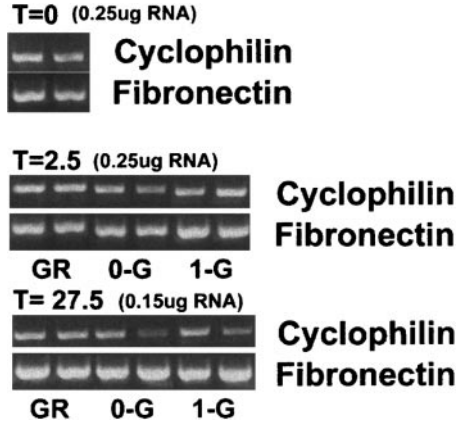

Figure 4. RT-PCR results for the amplification of FN mRNA. RNA was extracted from osteoblast sera activated on ground (GR) or in microgravity (0 *G*) or in an on-board 1-*G* centrifuge (1 *G*). Results are representative of four samples for each condition.

FN mRNA

To determine the level of regulation at which FN synthesis is controlled, FN mRNA synthesis was measured using RT-PCR (**Fig. 4**). A specific band corresponding to the expected FN PCR product was in high copy number relative to most of the other genes analyzed. This increase was more pronounced at the 27.5 h time point where we reduced the total amount of RNA in the RT-PCR reaction in order to remain in a linear amplification range. Final densitometry quantification was corrected to micrograms of RNA so both the 2.5 h and the 27.5 h time points could be directly compared.

As shown in **Figure 5**, 2.5 h after initiation of osteoblast growth in microgravity, there was a small but significant change in the total FN mRNA synthesis between the 0-*G* and 1-*G* samples, with mRNA synthesis for FN in the 0-*G* samples lower than 1 *G*. There was a trend for the GR samples to be higher, but it was not significant due to outlying data points. In contrast, at the 27.5 h time point, there is no significant difference in FN mRNA synthesis between samples grown in microgravity and those grown in a 1-*G* environment. In addition, at this later time point (27.5 h) the FN message was over twofold higher than at the 2.5 h timepoint.

DISCUSSION

The loss of bone in spaceflight is thought to be due to a lack of osteoblast activity and possibly to a defect

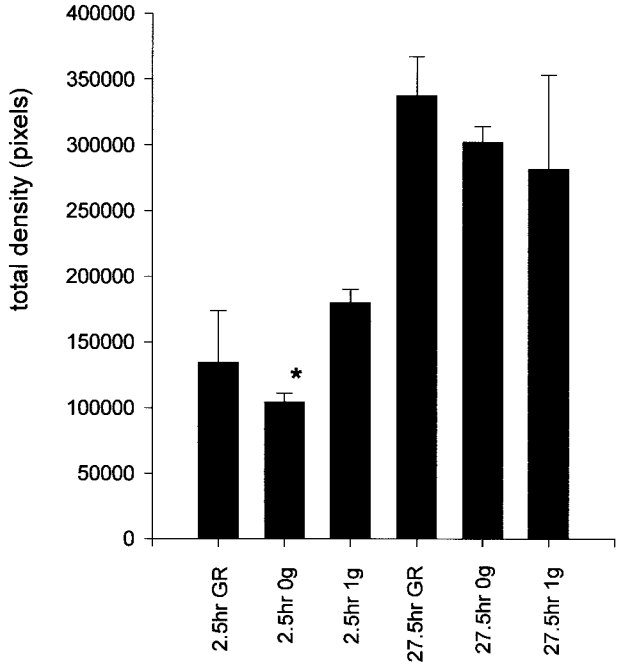

Figure 5. Quantification of FN RT-PCR. PCR bands from 2.5 h and 27.5 h samples were digitized and quantified as described in Materials and Methods. Total pixel density was corrected for the amount of RNA added to the RT reaction. For each condition the sample size was $n = 4$ and presented as total pixels ± SD. In the 2.5 h samples there was a significant difference between the 0-*G* and 1-*G* samples ($P < 0.001$). There was approximately a twofold increase in FN message in the 27.5 h samples compared to the 2.5-h samples with no significant difference between the gravity conditions.

in morphological assembly of the ECM. Previous work has demonstrated a cell shape change in osteoblasts exposed to microgravity after 15 or more parabolic weightless events or as long as 4 days. Change in cell shape is heterogeneous, with a portion of the cells having nuclear elongation and a smaller area of contact with the surface (9, 10). Experiments on STS-56 demonstrated that osteoblast cells had a change in cell shape after 4 days of weightlessness (9) and, more recently, experiments by Kacena et al. (11) demonstrated a significant change in osteoblast cell shape in microgravity as early as 3 h and as late as 3 days. None of these experiments had the convenience of an on-board 1-*G* centrifuge. We have recently published evidence (12) that nuclear morphology as well as the cell morphology was altered in microgravity when compared to on on-board 1-*G* control in these Biorack experiments. The changes in actin cytoskeleton and the nucleus were heterologous in 0-*G* with a portion of the cells showing nuclear elongation. In this study, in an attempt to explain the changed osteoblast cell shape, we examined the ECM FN on the Biorack samples.

FN plays a crucial role in growth and differentiation of osteoblasts (28) and is crucial for normal growth and cell spreading (15). We considered FN as a potential moiety for controlling the altered cell shape seen in microgravity. This study was undertaken to assess the role of FN in alterations of cell shape and cell matrix under microgravity conditions. By launching quiescent osteoblasts and activating and collecting them in microgravity, we were able to analyze gene activation and FN metabolism under microgravity conditions. In addition, for the first time, a total study of cell regulation of protein message, polypeptide synthesis, and immunolocalization of matrix was accomplished in spaceflight samples.

Because protein and RNA were extracted from the same samples, we were able to analyze both the transcriptional and translational control of FN. Although there was a slight and significant change in both messenger RNA and protein 2.5 h after sera activation, at later timepoints FN was not regulated differently in microgravity. We do not know why the earlier time points show significantly lower levels of both FN mRNA and protein synthesis but it is possible that a slow response to sera activation could be causing a lag in response since it is known that growth factor-induced signal transduction is sensitive to gravity (28). The presence of *de novo* synthesis of FN by MC3T3-E1 osteoblast is supported by results that show an accumulation of both FN message (more than twofold) and protein (more than fourfold) during the 25-h incubation period between the early and late collection times. This finding is consistent with reports of others which show that FN protein, unlike other osteoblast matrix molecules, is synthesized throughout osteoblast growth and differentiation (29). These data suggest that microgravity-induced cell morphology alterations are not caused by an aberration in the FN portion of the ECM. Extrapolation of our findings also suggests that changes in FN are not a causal factor in the loss of osteoblast growth in spaceflight. However, it is possible that FN binding and receptor-mediated signaling may be affected in microgravity and play a role in other altered responses.

The analysis of FN matrix from parallel samples fixed in flight for immunofluorescence localization studies confirmed the Western blot analysis showing that FN protein synthesis was not affected by microgravity. In addition, these data reveal that FN protein had been synthesized and exported out of the cells. The results also demonstrate that typical organization of the matrix was occurring. Finally, our data shows that 1 day after sera activation in 0-*G*, the samples have an overall regulation of FN that is indistinguishable from 1-*G* on-board controls or ground controls. Taken together, it is likely that changes in bone matrix formation, cell shape, and loss of bone growth previously observed in micro-

gravity are not dependent on changes in FN regulation. FJ

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