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ORIGINAL ARTICLE

Alteration of cell cytoskeleton and functions of cell recovery of normal human osteoblast cells caused by factors associated with real space flight

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Abstract

Experiments involving short-term space flight have shown an adverse effect on the physiology, morphology and functions of cells investigated. The causes for this effect on cells are: microgravity, temperature fluctuations, mechanical stress, hypergravity, nutrient restriction and others. However, the extent to which these adverse effects can be repaired by short-term space flown cells when re-cultured in conditions of normal gravity remains unclear. Therefore this study aimed to investigate the effect of short-term spaceflight on cytoskeleton distribution and recovery of cell functions of normal human osteoblast cells. The ultrastructure was evaluated using ESEM. Fluorescent staining was done using Hoechst, Mito Tracker CMXRos and Tubulin Tracker Green for cytoskeleton. Gene expression of cell functions was quantified using qPCR. As a result, recovered cells did not show any apoptotic markers when compared with control. Tubulin volume density ($p<0.001$) was decreased significantly when compared to control, while mitochondria volume density was insignificantly elevated. Gene expression for IL-6 ($p<0.05$) and sVCAM-1 ($p<0.001$) was significantly decreased while alkaline phosphatase ($p<0.001$), osteocalcin and sICAM ($p<0.05$) were significantly increased in the recovered cells compared to the control ones. The changes in gene and protein expression of collagen 1A, osteonectin, osteoprotegerin and beta-actin, caused by short-term spaceflight, were statistically not significant. These data indicate that short term space flight causes morphological changes in osteoblast cells which are consistent with hypertrophy, reduced cell differentiation and increased release of monocyte attracting proteins. The long-term effect of these changes on bone density and remodeling requires more detailed studies.

Key words: osteoblasts, microgravity, imaging, bone markers

INTRODUCTION

When no longer stressed by gravity, bones demineralize, losing both calcium and strength.[1] Numerous investigations have demonstrated data on different aspects of adaptation of the bone tissue to microgravity and other space flight-related factors, like hypergravitation, vibration and hypokinesia.[2-6] The effect of weightlessness on cultured osteoblasts and their precursors was described by a number of investigators,[7-9] most of whom were using simulated hypogravity for their experiments.[10-15] It was shown that in space the proliferation of osteoblasts is inhibited, differentiation is delayed and expression of genes controlling differentiation is reduced.[2,3,4,16,17,18] The same trend was discovered in experiments with simulated microgravity.[15,19,20,21] Space-flown pre-osteoblasts followed a slower progression toward a differentiated function.[8] The apoptosis rate was shown to increase in simulated microgravity in osteoblast-like cells ROS 17/2.8 due to damage to the cytoskeleton,[10] while other investigators[15] did not observe apoptosis in cultured osteoblasts exposed to weightlessness.

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Contradictory data were presented regarding secretory activity of the space-flown osteoblasts. Some authors demonstrated reduced mRNA alkaline phosphatase expression,[22] while others showed that its activity did not change.[7,17] On the contrary, other authors demonstrated a double increase of alkaline phosphatase activity and expression after 6 days of real space flight.[23]

In experiments with stimulated microgravity some authors also showed decrease of the expression of mRNA alkaline phosphatase[15,24] while some researchers showed that the expression did not change while the activity was suppressed[14] and yet others showed that both activity and expression were enhanced, combined with increased expression of osteopontin and BMP-4, thus demonstrating marked osteoblastic phenotype.[19]

In real space flight experiments[8,22] and simulated microgravity[15,24] collagen type 1 protein and gene expression were shown to decrease, while others reported that it did not change.[7] IL-6 was shown to decrease in space but normalized within 24 hours of gravity.[7] TGF beta and its mRNA were demonstrated to decrease during space flight.[3,7,25] Expression and secretion of osteocalcin was shown to decrease in simulated microgravity[14,15,24] and real space environment, [2,4,8,22] while others did not find any changes in the level of osteocalcin mRNA in space flight[7] or found it increased in modeled microgravity.[19] PGE2 was shown to go up,[3] down[16] or remain unchanged.[7] Only cytokines and skeletal growth factors were found to be changed under microgravity, while matrix proteins like osteocalcin and collagen type 1 did not change.[7]

Gravity forces appear to be required for the osteoblasts to maintain a stable cytoskeleton. It is difficult to evaluate cytoskeletal rearrangement in microgravity because of the complexity of cytoskeletal structure.[24,26] Further investigations are required to explain more thoroughly the underlying mechanisms.[27,28] Cytoskeletal changes were considered to be adaptive to the reduced load on the weight-bearing regions of the skeleton.[29] It is important to develop measures to modulate biomarkers of bone metabolism before, during and after the space flight, i.e., to promote osteoblast differentiation and to prevent bone loss.[21,30] Most of the cited sources refer to the changes of osteoblasts. Few investigations are focused on osteoclasts' changes in microgravity or changes of both.[31] It was shown that genes responsible for maturation of osteoclasts multiply their expression in space.[32]

Very few investigations based on microscopic imaging were done on osteoblasts and bone tissue after space flights.[4,8,22,33,34] They demonstrated changes of cell shape, cytoskeletal and adhesion structures together with signaling transduction pathways, and gene expression leading to possibly altered growth/differentiation. *In vitro* studies carried out on osteoblastic cell cultures in space showing changes in cell shape suggest that cell attachment structures as well as cytoskeleton reorganization might be involved in adaptation to microgravity.

While there are several papers demonstrating change in the shape of osteoblasts and osteocytes *in vitro*, osteocytes were recently shown for the first time to acquire a round shape *in vivo* as a result of space-related morphological alterations.[6] Regarding cultured osteoblasts, they were shown to decrease their size[16] due to retraction of their cytoplasm and to become rounder and covered with microvilli.[23] Nuclei of the cells become elongated.[16] A decrease in the expression of collagen type1, alkaline phosphatase, osteocalcin and reduced proliferative activity was explained by the change in the cells' shape.[24]

Few of the investigators used electron microscopy to detect alterations in osteoblastic cells under real or modeled microgravity conditions.[5,13,18,31,34,35]

A new chapter in research on space-related changes in the bone tissue was opened by experiments with transgenic animals: the ones with an overexpression of pleiotrophin[6] exerting a positive effect on bone turnover; and lacking calponin H1[36] which suppresses bone formation, thus demonstrating the possibility to control bone loss during long-term space flights and some other conditions. Attempts were undertaken to forecast development of osteopenia in potential candidates for space flight.[37,38]

Most of the papers on outer space flight-related bone tissue alterations describe immediate post-flight changes of the osteoblasts, and very few of them evaluate long-term consequences of the microgravity exposure for the bone cells, while it was commented[39] that bone recovery from the weightlessness exposure lasts longer then the duration of this exposure. Few researchers followed up changes of the bone cells and tissue during the post-flight period. Osteoblasts sampled from the monkeys after the outer space flight showed inhibited proliferation and no changes of osteocalcin and alkaline phosphatase expression after 14 days of culturing.[17] The human study

showed that bone mineral density in astronauts was not recovered even after 6 months at normal gravity.[29] The capability of cultured osteoblasts to self-repair in gravity remains unclear. No "recovery" space flight experiments have been performed using primary normal human osteoblast cells. Therefore, we undertook this investigation to examine the association between ultrastructure, cytoskeleton damage and function of the space-flown human osteoblasts after their re-adaptation to gravity.

The objective of the present investigation is to examine the association of osteoblast cytoskeleton, adhesion molecules and protein bone marker function at the cell culture level after short term space flight.

MATERIALS AND METHOD

Cell culture

The 500,000 cells of Primary Normal Human Osteoblast cells (NHOst; Lonza, USA) at passage 6 were cultured on microcarriers (cytodex 3; GE Healthcare, USA) and kept in fluid processing apparatus. Cells were flown at the international space station for 10 days before they were brought down to earth at day 12 as described earlier.[40] The surviving cells were detached from the cytodex 3 and re-cultured on normal flasks until their cell number were sufficient for cytoskeleton, cell motility, gene and protein expression study.

Scanning electron microscopy of normal and space-flown osteoblasts

NHOst and space flown, microgravity recovered osteoblast cells (MRO) cells were cultured on a thermanox cover slip (NUNC, USA) for 10 days. Cells on the cover slip were fixed with 2% osmium tetraoxide in PBS and dehydrated by serials of ethanol. After dehydration, the cells were critical-point dried with CO_2, coated with gold and examined under a scanning electron microscope Quanta FEG 200F from FEI.

Cytoskeleton and Apoptosis Staining

NHOst and MRO were cultured on glass bottom Petri dishes (NUNC) at a density of 2000 cells/dish. After 24 hours the cells were washed with PBS and directly stained with Mitochondria and Tubulin Tracker (Invitrogen, USA) according to the manufacturer's recommendation, or cells were stained with Acridine orange/Propidium Iodide for apoptosis investigation.

Cells were incubated at room temperature (20-25^{o}C) in the dark for 15 minutes with gentle shaking; fluorescence-stained cells were examined under the inverted Confocal Laser Scanning Microscope (Leica TCS SP5), which is equipped with a laser confocal system comprised of a 405 Diode laser, argon laser, HeNe 543 & 633 and four photomultiplier tubes. Image processing was carried out with LAS AF software.

NHOst and MRO double-immunostained for mitochondria and tubulin and counterstained by Hoechst (nucleus) were photographed using a 40x objective. The captured images of cells were saved under .tiff extension, recorded for blind analysis by the two investigators and quantified using image analysis. The contours of the integral cells and their nuclei were outlined, images were calibrated and measurements of the cellular and nuclear area and diameters (maximal and minimal) were performed using Leica QWin software (Leica imaging systems, Cambridge, UK). The area of intracellular fluorescence for each cell (red for mitochondria and green for tubulin) was quantified.

The measurements were processed using Excel software version 3.05 (GraphPad Software, San Diego, CA, USA) with subsequent estimation of the volume density of the intracellular fluorescence for mitochondria and tubulin staining, rugosity index of the cells (estimated as FF=4pi x area/square perimeter) and the ellipticity index of the nuclei (estimated as max diameter to min diameter ratio). Mean area (mcm^2), diameters (mcm), of the cells and their nuclei and the volume density (%) of the mitochondria and tubulin and their mean errors were calculated. The significance of the differences in these parameters in NHOst and MRO was assessed by the two-tailed Student's test. The level of significance was set at $p < 0.05$.

Gene Expression

Total RNA was obtained using Allprep DNA/RNA/Protein kit from Qiagen (Germany). Total RNA quality was assessed and RNA concentration was determined using the Bioanalyzer (Agilent, USA). 40ng/ul of total RNA was reverse transcribed to cDNA by using Sensiscript kit, Qiagen. Then 2ul of cDNA was mixed with SYBR Green Master Mix from Bio-Rad (USA) and primers for target and reference gene (Table 1 for a list of genes and sequences). QPCR was done using the MyiQ system from Bio-Rad (USA) and gene expression analysis was calculated using iQ5 software (Bio-Rad,

Table 1. Gene list and primer sequence that have been used in qPCR

Gene name	Primer sequence
GAPDH	Forward 5' TGCACCACCAACTGCTTAGC 3' Reverse 5' GGCATGGACTGTGGTCATGAG 3'
Alkaline Phosphatase	Forward 5' ATGGGATGGGGTCTCCACA 3' Reverse 5' CCACGAAGGGGAACTTGTC 3'
Collagen type 1 alpha	Forward 5' GTCGAGGGCCAAGACGAAG 3' Reverse 5' CAGATCACGTCATCGCACAAC 3'
Osteocalcin	Forward 5' CGCAGCCACCGAGACACCAT 3' Reverse 5' GCCAGCAGAGCGACACCCTA 3'
IL6	Forward: 5' AAC CTG ATC CTT CCA AAG ATG G 3' Reverse: 5' TCT GGC TTG TTC CTC ACT ACT 3'

USA) with $^{\Delta\Delta}C_t$ of the sample normalized against control and reference gene (GAPDH).

RESULTS

Scanning electron microscopy of NHOst and MRO

Scanning electron microscopy (SEM) of the control cells demonstrated their regular, mostly spindle-like shape, centrally located nuclei with prominent nucleoli, numerous pores rather evenly distributed on the surface of the cells and visible threads of cytoskeleton running parallel to the long axis of the cell (Fig. 1a). MRO cells exhibited enlarged size, irregular shape with increased amount of processes and outgrowths, vast cytoplasm and eccentrical nuclei (Fig.1b, c). Intermingled threads of cytoskeleton were thinner and less prominent than in the control (Fig.1d). Numerous microvilli were unevenly distributed on the surface of the cells with vast areas devoid of surface specializations (Fig.1d, e). Numerous pores are concentrated in the perinuclear zone and are almost absent in the cytolemma of the processes and outgrowths (Fig.1b, c, d, e). Large pores and rare blebs are visible in the perikarions of the cells (Fig.1f). Thus SEM provided information regarding increased size of the MRO, their irregular shape due to numerous processes and outgrowths, increased amount and redistribution of blebs and pores on the cellular surface, uneven distribution of the surface specializations with "bare areas" mainly in the perinuclear region, and changes of the cytoskeleton in the MRO cells compared to the NHOst.

Fluorescence microscopy of NHOst and MRO

NHOst were mostly spindle-shaped with the two short processes. Fewer cells were round or triangular with three and more processes, some of which were quite long. A few cells contained eccentric nuclei. NHOst were highly positive for mitochondria and tubulin markers. Red and green fluorescence was evenly distributed in most of the cells, though in some of them mitochondria and tubulin markers were polarized (Fig.2a).

Appearance of the microgravity recovered cells differed considerably from the normal ones (Fig.2 b, c, d). The size of the cells was notably increased indicating their hypertrophy; the contours became intricate with many processes of different length and shape, including confluent ones. Staining for mitochondria and tubulin became polarized, as bundles of the tubulin-positive structures mostly occupied the periphery of the cells. Staining by propidium iodide and acridine orange showed a negative result for apoptosis in MRO cells (Fig.3). The results of the image analysis are shown in Fig.4. As it follows from Fig.4a, all morphometric parameters of the MRO, like area, maximal and minimal diameter, were significantly higher than in the NHOst ($p<0.001$). They were increased accordingly to 17.44-fold, 4.75-fold and 3.0-fold of control levels. Rugosity index of the post-flight cells was 1.92 times lower than in the normal cells ($p<0.001$) indicating increased irregularity of their cell surface.

The area, maximal diameter and minimal diameter of the nuclei (Fig.4b) were also meaningfully higher in the MRO with different levels of significance ($p<0.001$). They were increased accordingly to 3.25-fold, 1.79-fold and 1.77-fold of control levels. Ellipticity of

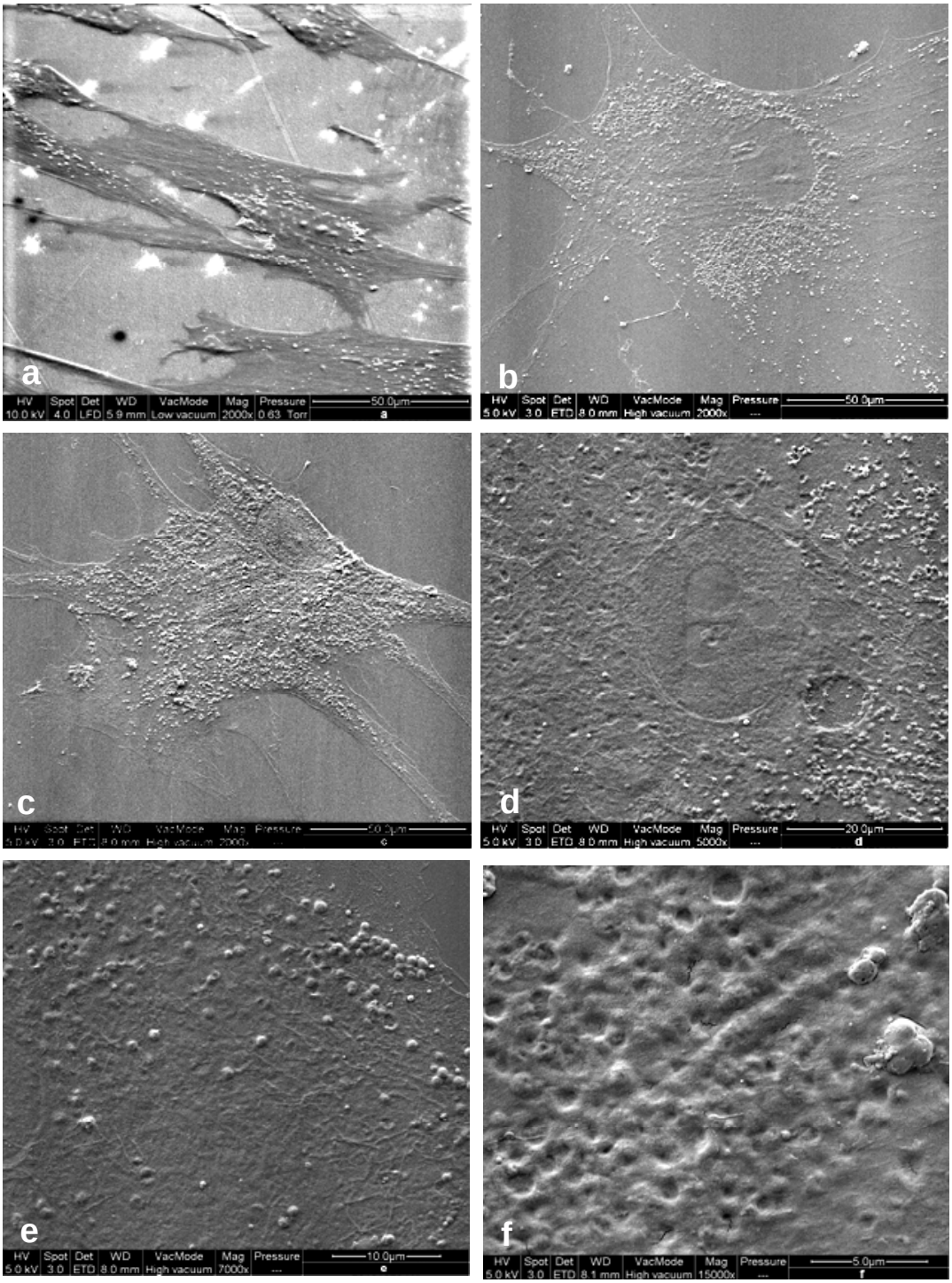

FIG. 1 Scanning electron microscopy of NHOst and MRO. Shown are NHOst control samples (a, b) and MRO cells (c, d, e, f). The pictures were taken with a Quanta FEG 200F at high vacuum and 5000, 7000 and 15000 times magnifications.

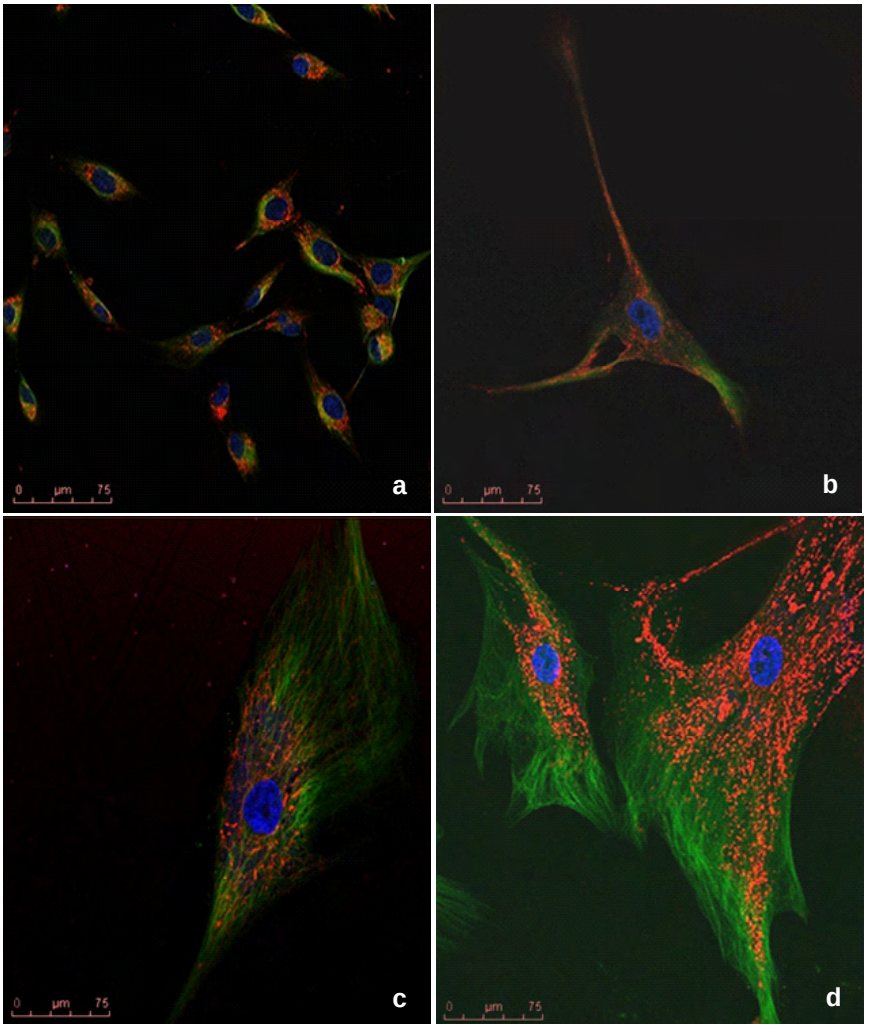

FIG. 2 Fluorescence confocal laser scanning microscopy of NHOst and MRO. Shown are NHOst control cells (a) and MRO cells (b, c, d). The cells were fixed and stained with Mitochondria (red) and Tublin Tracker (green) as well as Hoechst 33342 (blue) for the nucleus. The cells were viewed with CLSM under 40x magnification (Scale bar: 75 µm).

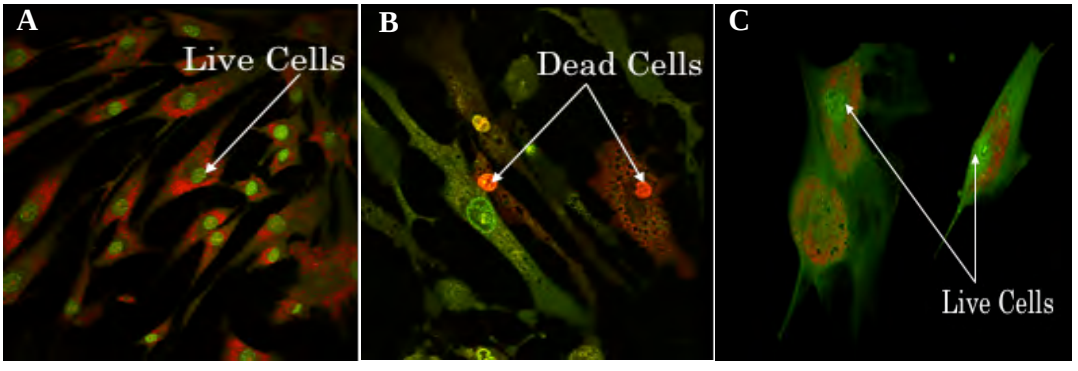

FIG. 3 Fluorescence staining for apoptosis using Propodium iodide/Acridine orange. NHOst control cells (a) and MRO cells (c) were used for apoptosis staining. The nuclei of live cells stained with green color. (a,c) while in damaged cells the nuclei stained red (b). As positive control cells served NHOst cells exposed to UV light for 2 hours. MRO cells (c) did not show any apoptotic markers. The samples were scanned with a CLM at 40x times magnification.

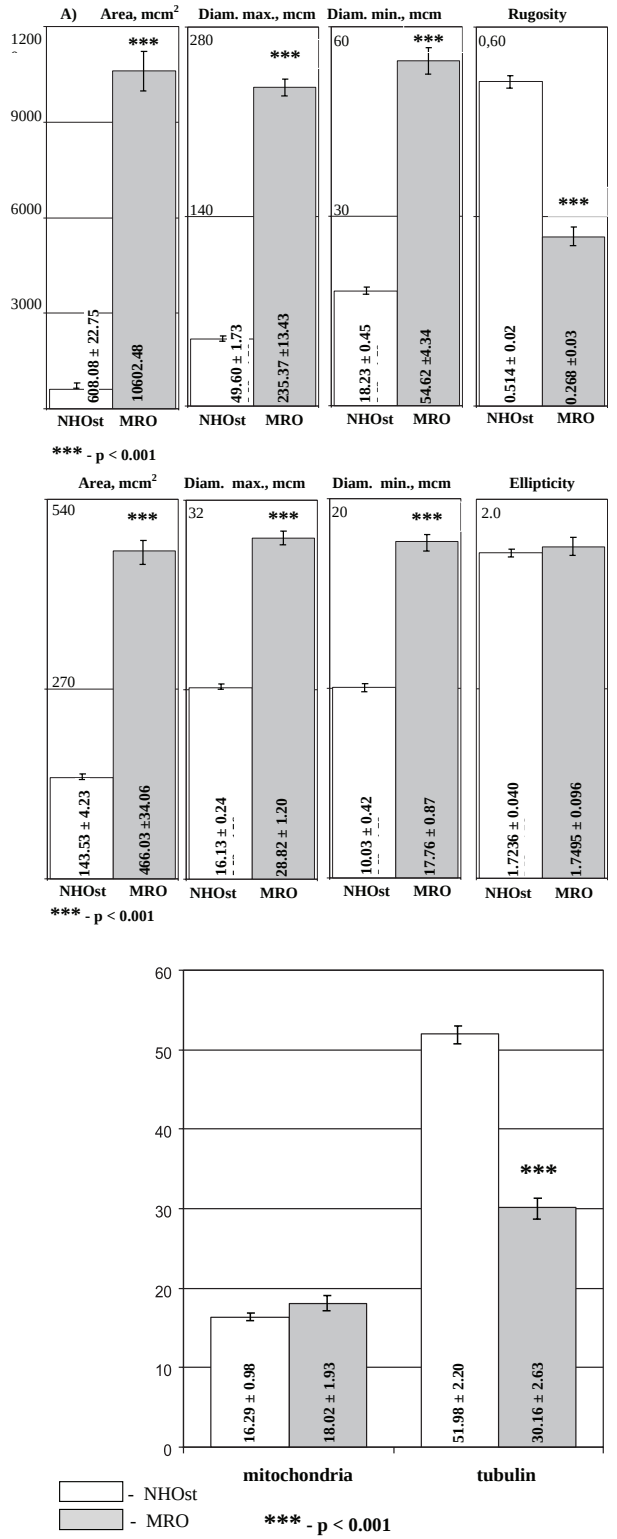

FIG. 4 Image analysis for Fluorescence stained NHO and MRO cells. Calculated are the cell parameters (a) for NHOst control cells and MRO cells with regards to area, maximum and minimum diameter and rugosity (M+/-SE) as well as for the nucleus (b) with regards to area, maximum and minimum diameter and ellipticity. Figure 4c shows the volume intensity for mitochondria and tubulin staining (M+/-SE).

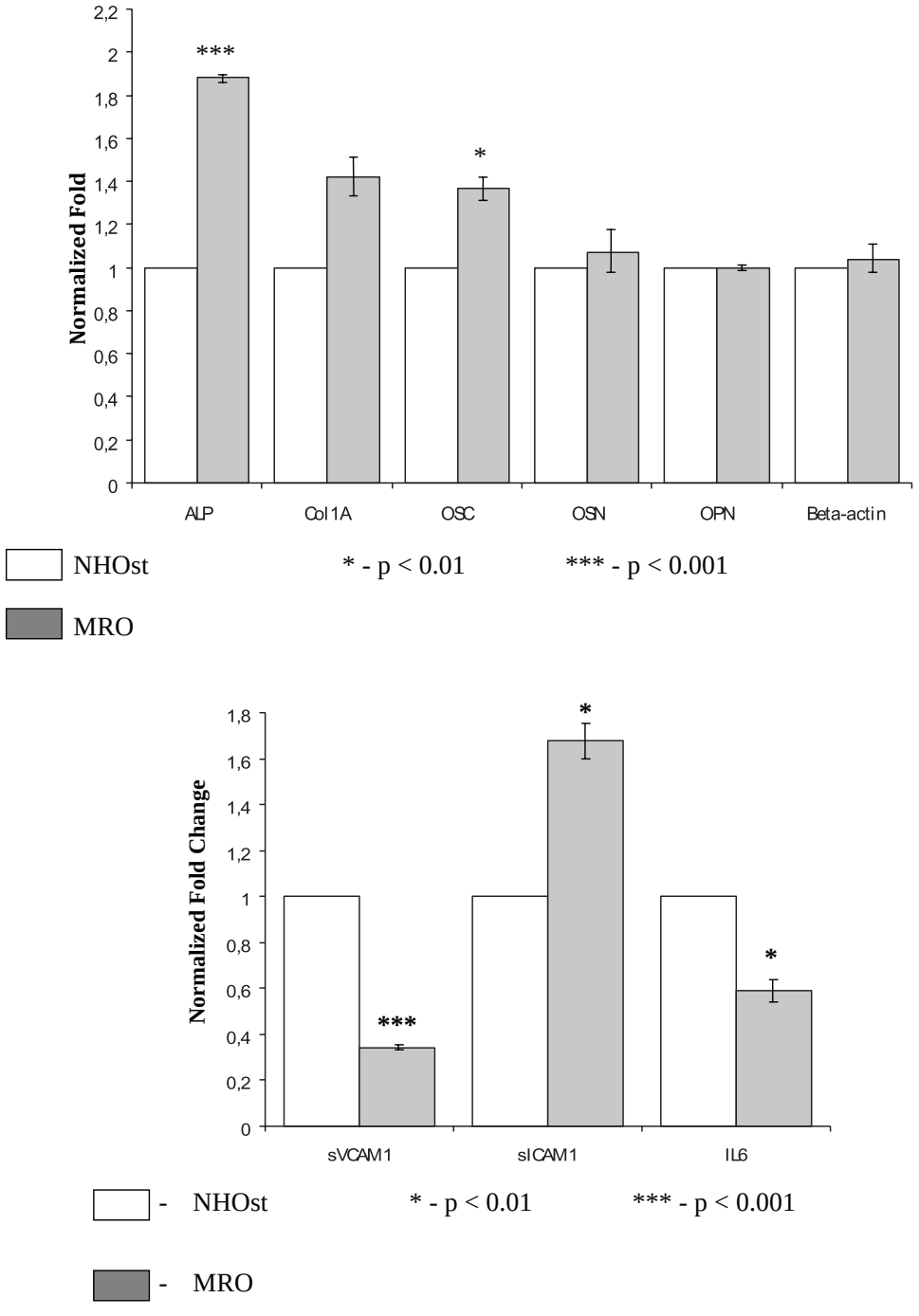

FIG. 5. Gene expression of bone metabolism markers, adhesion molecules and interleukin 6 in NHOst and MRO. Total RNA extracted from NHOst and MRO cells was reverse transcribed and amplified with real-time PCR using specific primer sequences (Table 1). The gene expression fold changes were normalised against GAPDH (reference gene) and NHOst control. Data are expressed as mean ± SD ($n = 6$). Statistical analysis by Independent-samples T test showed significant differences. *: $P < 0.01$; ***: $P < 0.001$.

the nuclei did not differ between MRO and NHOst.

Volume density (Fig.4c) of the red fluorescence (staining for mitochondria) was slightly increased in the MRO cells compared to the NHOst, while the volume density of the green fluorescence (staining for tubulin) was significantly lower in the MRO ($p<0.001$, 1.72-fold decrease).

These findings suggest that increased irregularity of the cell surface in the MRO may be explained by cytoskeleton damage under microgravity conditions indicated by the decreased volume density of the tubulin-positive microtubules and the SEM images of the MRO. Enlargement of the MRO is a result of their hypertrophy as indicated by unchanged volume density of mitochondria which would be reduced in case of cell size increase through other reasons. In certain cases decreased volume density of the organelles is indicative of cytoplasmic edema, but as a decrease in the tubulin-positive structures in the MRO is accompanied by an increased volume density of mitochondria, it may be interpreted as disturbance of the microtubular network in the hypertrophied MRO cells.

Hypertrophy of MRO and increased volume density of the mitochondria in them may be the mechanism accounting for the adaptation of the cells to the space conditions.

Gene expression of bone and cell-cell interactions markers for extracellular matrix formation

We found (Fig.5a) that the alkaline phosphatase (ALP) expression was significantly increased in the MRO cells ($p<0.001$), which shows that the cells undergo a matrix maturation phase followed by expression of collagen type 1 (Col 1) and osteocalcin (OSC), the latter also being significantly increased ($p<0.05$).

ICAM1 showed a significant increase in gene expression ($p<0.05$) while gene expression of sVCAM1 ($p<0.001$) and IL6 ($p<0.05$) was significantly decreased. The changes in the other gene expressions of cell functions (collagen 1, osteonectin, osteoprotegerin, beta-actin) caused by short-term spaceflight were statistically not significant.

DISCUSSION

The absence of gravitational forces therefore causes both an increase in bone resorption by osteoclasts, and a decrease in osteoblast cellular integrity.[31]

Changes of the microtubules during exposure to microgravity are a long known phenomenon. The fluorescent microscopic images of microgravity recovered osteoblasts were obtained to describe shape and size of the cells, their nuclei and distribution of mitochondria and microtubules in the cytoplasm under normal and space flight conditions. Our findings suggest that the specified changes in the cellular and nuclear size and contours, and volume density of the microtubules in MRO cells reveal cytoskeletal alteration of the cells adjusting to the space conditions. Image analysis of the cells stained for mitochondria and tubulin together with SEM images evaluation are useful for description of the adaptational changes in the osteoblasts recovered after the outer space flight.

Alteration occurring in gene expression of adhesion molecules is tightly regulated by the cell cytoskeleton and associated directly with the cell cycle. Therefore, as a result the cells are not able to proliferate well due to the arrest occurred at the GO/G1 phase of the cell cycle. However, based on the results obtained, these effects could not be overcome by MRO cells after a certain period in normal gravity conditions but by extending the recovery time, the chances for MRO cells to return to a normal cell cycles are high. These results disclose the specific target of genes for therapeutic treatment for future space exploration missions.

Interestingly, the increase of ICAM1 and osteocalcin gene expression indicates that the MRO are sending out signals to attract monocytes. The significant decrease of sVCAM1 and IL6 gene expression seems to be however contradictory to the previous finding. It was earlier suggested[41] that adhesion molecules and IL6 interplay in the process of osteoclastogenesis via LFA-1/ICAM1 and VLA-4/VCAM1 pathways as a regulator for production of osteoclast differentiation.

In our research MRO displayed a high expression of osteocalcin together with the increased expression of ICAM1. Research has shown that both are involved in attracting monocytes to the bone. These monocytes then will differentiate into osteoclasts. Additionally it has been shown that osteoclasts require high osteocalcin content to reabsorb bone effectively. In addition to its role in bone metabolism, osteocalcin also has an endocrine role in which it increases insulin release and target cell sensitivity towards insulin.[42] This aspect of osteocalcin function has been highly neglected. From our results we cannot draw any conclusions towards the endocrine function of osteocalcin as this is

associated with the undercarboxylated status of osteocalcin which we did not measure. However it is an interesting link which requires more investigations.

CONCLUSION

As gravity forces are required for the osteoblasts to maintain their stable cytoskeleton, microgravity results in its disintegration, which may explain dramatic changes in the size and shape of the cells and their surface specializations. MRO cells underwent premature aging, but did not initiate the cell death program.

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