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Characterization of the Osteoblast-Like Cell Phenotype Under Microgravity Conditions in the NASA-Approved Rotating Wall Vessel Bioreactor (RWV)

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Abstract Weightlessness induces bone loss in humans and animal models. We employed the NASA-approved Rotating Wall Vessel bioreactor (RWV) to develop osteoblast-like cell cultures under microgravity and evaluate osteoblast phenotype and cell function. Rat osteoblast-like cell line (ROS.SMER#14) was grown in the RWV at a calculated gravity of 0.008*g*. For comparison, aliquots of cells were grown in conventional tissue culture dishes or in Non-Rotating Wall Vessels (N-RWV) maintained at unit gravity. In RWV, osteoblasts showed high levels of alkaline phosphatase expression and activity, and elevated expression of osteopontin, osteocalcin, and bone morphogenetic protein 4 (BMP-4). In contrast, the expression of osteonectin, bone sialoprotein II and BMP-2 were unaltered compared to cells in conventional culture conditions. These observations are consistent with a marked osteoblast phenotype. However, we observed that in RWV osteoblasts showed reduced proliferation. Furthermore, DNA nucleosome-size fragmentation was revealed both morphologically, by in situ staining with the Thymine-Adenine binding dye bis-benzimide, and electrophoretically, by DNA laddering. Surprisingly, no p53, nor bcl-2/bax, nor caspase 8 pathways were activated by microgravity, therefore the intracellular cascade leading to programmed cell death remains to be elucidated. Finally, consistent with an osteoclast-stimulating effect by microgravity, osteoblasts cultured in RWV showed upregulation of interleukin-6 (IL-6) mRNA, and IL-6 proved to be active at stimulating osteoclast formation and resorbing activity in vitro. We conclude that under microgravity, reduced osteoblast life span and enhanced IL-6 expression may result in inefficient osteoblast- and increased osteoclast-activity, respectively, thus potentially contributing to bone loss in individuals subjected to weightlessness. J. Cell. Biochem. 85: 167–179, 2002.

Key words: osteoblast; microgravity; apoptosis; bone remodelling

Cells are subjected to a delicate balance throughout our life due to a variety of factors that contribute to the maintenance of tissue homeostasis. Numerous molecular mechanisms are claimed to determine tissue development and equilibrated functions, among which physical forces are known to largely affect body building. The general belief is that each tissue in the body suffers abnormal alteration when subjected to reduced mechanical strains [West, 2000]. However, those apparatuses that contribute to locomotion (i.e., somatic musculature

Nadia Rucci and Silvia Migliaccio contributed equally to this work.

Abbreviations used: ALP, alkaline phosphatase activity; BMP-2, bone morphogenetic protein 2; BMP-4, bone morphogenetic protein 4; BSP II, bone sialoprotein II; DMEM-F12, Dulbecco's modified minimum essential medium-F12; ECL, enhanced chemiluminescence; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IL-1β, interleukin-1β; IL-6, interleukin-6; M-MLV, moloney murine leukemia virus; ODF, osteoclast differentiation factor; ON, osteonectin; OPN, osteopontin; OPGL, osteoprotegerin ligand; OCN, osteocalcin; PBS, phosphate buffered saline; RANK, receptor activator of NF-kB; RANKL, receptor activator of NF-kB ligand; RT-PCR, reverse transcriptase-polymerase chain reaction; RWV, rotating wall vessel; SDS, sodium dodecyl sulfate; TRANCE, TNF-related activation-induced cytokine.

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and skeleton) are more likely affected by mechanic responses [Frost et al., 1998]. For instance, limb unloading by pathologic nerve degeneration in humans or by experimental neurotomy in animal models leads to muscle atrophy and local bone loss [Globus et al., 1986].

Space-flights have largely contributed to our knowledge on the effects of mechanical unloading on body tissues. Astronauts and experimental animals develop severe, generalized osteopenia during their journeys around the Earth [Morey and Baylink, 1978; Vico et al., 2000]. The underlying molecular mechanisms, however, remain to be fully elucidated.

Experiments in space offer the advantage of overall observations of changes occurring in the whole body. However, their pitfall meets with the unavailability of controlled tissue and cell culture conditions for molecular investigations, particularly for short-term experiments. On ground, microgravity can be simulated using vector-free horizontal clinostats [Sarkar et al., 2000], or by hindlimb unloading [Van Loon et al., 1995]. Recently, NASA has designed a cell culture double-walled rotating bioreactor to be used for tissue cultures both on Earth and in space-flights, named Rotating Wall Vessel bioreactor (RWV) [Freed et al., 1999; Pellis, 1999; Jessup and Pellis, 2001]. Suspended cells rotate in the vessels of the RWV as a solid body with minimal disruptive shears, and the cells maintain their relative position for long times allowing them to touch one another. Chamber rotation subjects the cells to a constantly changing angular gravity vector. Constant randomization of the normal gravity vector subjects the cells to a state of simulated free fall, similar to microgravity conditions [Duray et al., 1997; Freed et al., 1997].

Osteoblasts are bone forming cells whose task is to build the skeleton and regulate osteoclast differentiation and activity [Lian et al., 1999]. Physiological strain plays an important role in maintaining the normal function and metabolism of osteoblasts. A few studies have been undertaken so far to elucidate the behavior of osteoblasts subjected to microgravity conditions, and the mechanisms whereby changes in mechanical forces are transduced into the cell and modify osteoblast function have not yet been understood [Morey and Baylink, 1978; Marie et al., 2000; Sarkar et al., 2000]. To elucidate whether the osteoblast phenotype is maintained under microgravity conditions and whether changes occur compared to conventional tissue cultures, we have used the NASA RWV, where the ROS.SMER#14 cells were cultured and evaluated for their specific osteoblast markers.

MATERIALS AND METHODS

Materials

DMEM-F12 phenol red free, FCS, penicillin, streptomycin, and trypsin were from GIBCO (Uxbridge, UK). Sterile plasticware was purchased from Falcon Becton-Dickinson (Cowley, Oxford, UK) or Costar (Cambridge, MA). HRP-conjugated antibodies were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Antibodies L-123 to osteopontin (OPN), BON-I to osteonectin (ON) and LF-87 to bone sialoprotein II (BSP-II) [Fisher et al., 1995] were kindly donated by Dr. Larry Fisher (Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD). The nuclear and cytoplasmic extraction NE-PER kit was purchased from Pierce (Rockford, IL).The ECL kit was from Amersham Pharmacia Biotech (Milan, Italy). Primers and reagents for RT-PCR were from Promega (Milan, Italy). All the other reagents were of the purest grade from Sigma Aldrich, Co. (St. Louis, MO).

RWV

The RWV Bioreactor (model STLV, size 55 ml) was purchased from Synthecon CELLON S.ar.l (Strassen, Luxembourg). It consists of a cylindrical growth chamber that contains an inner co-rotating cylinder with a gas exchange membrane (Fig. 1). The fluid dynamic principles of the RWV Bioreactor allow oxygenation without turbolence, co-localization of particles with different sedimentation rates, and reduction of fluid shear forces. Cells and liquid culture media were placed in the space between the inner and the outer cylinders, and the assembled device was rotated about its longitudinal axis [Duray et al., 1997; Freed et al., 1997]

Cell Culture

For all the experiments, we used the ROS. SMER#14 cells, a rat osteoblast-like cell line developed in our laboratory [Migliaccio et al., 1992], expressing all the markers of the osteogenic phenotype [Migliaccio et al., 1992]. ROS.SMER#14 cells were cultured at a density

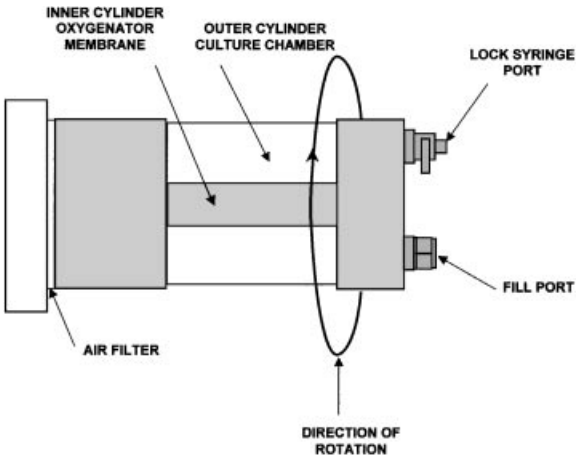

Fig. 1. Schematic drawing of the RWV. RWV consists of a cylindrical culture chamber containing an inner co-rotating cylinder with a gas exchange membrane through which an air pump draws incubator air. Cells and liquid media are placed in the space between the inner and the outer cylinders and the device is rotated about its horizontal axis.

of 100,000/ml in phenol red-free DMEM-F12 supplemented with 10% FCS, 100 U/ml penicillin, and 0.2 mg/ml streptomycin at 37°C and 5% CO_2 in a humidified incubator. Cells were cultured in three experimental conditions: in conventional tissue culture dishes and in bioreactor vessels not subjected to rotation (N-RWV), both maintained at unit gravity as well as in the RWV subjected to a rotation of 16 rpm, corresponding to a microgravity value of 0.008*g*. All cultures were carried out for 48 h.

Cell Viability Assay

Cell viability was evaluated by trypan blue exclusion test. Cells were collected, stained with 0.02% trypan blue, and counted by conventional light microscopy.

Alkaline Phosphatase (ALP) Activity

Cells were lysed in 0.1% SDS and ALP activity was evaluated biochemically in the cell extracts by the Sigma kit No.104, according to the manufacturer's instructions.

Western Blotting

For total protein extraction, cells were lysed in RIPA buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Nuclear and cytosol protein extraction was performed by the Pierce NE-PER kit no. 78833, according to the manufacturer's instructions. Protein content was measured by the Bradfod method. Proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes. Blots were probed with the primary antibody for 1 h at room temperature, washed, and incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Antibodies were diluted as reported in figure legends. Protein bands were revealed by ECL.

RT-PCR

Total RNA was extracted using the acid phenol technique [Chomczynsky and Sacchi, 1987]. One microgram of RNA was reverse transcribed using M-MLV reverse transcriptase and the equivalent of 0.1 μg was used for the PCR reactions. These were carried out in a final volume of 50 μl containing 200 μM of dNTPs, 1.5 mM $MgCl_2$, 10 pM of each primer, and 1 U Taq-DNA-polymerase. PCR conditions and primer pairs used are listed in Table I. For quantitative analysis, primers for the housekeeping gene GAPDH were used along with the primers for the gene being analyzed. PCR-amplified products were analyzed on 1.5% agarose gel containing ethidium bromide.

Morphology

Cells were fixed in 3% paraformaldehyde in PBS, washed, and observed by phase contrast microscopy in a Zeiss Axioplan microscope.

Fluorescence Microscopy

Cells were collected, attached to glass slides by cytospin procedure, fixed in 3% paraformaldehyde in PBS, washed in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 3 min at 0°C. Microfilaments were decorated by incubation with 10 μg/ml rhodamine thiocyanate-conjugated phalloidin. Cells were then observed by conventional epifluorescence in a Zeiss Axioplan microscope.

DNA Staining

Bis-benzimide, a dye that specifically binds the Adenine-Thymine sites, was used for DNA staining. Cells were collected, attached to glass slides by cytospin procedure, and fixed in Carnoy's fixative (methanol-glacial acetic acid, 3:1). Slides were then incubated for 30 min in 0.5 μg/ml bisbenzimide, rinsed twice in distilled water, mounted in glycerol-PBS 1:1, and observed by conventional epifluorescence microscopy.

TABLE I. Primer Pairs and PCR Conditions

Gene	Primers	PCR conditions	bp	References
ALP	Fw: 5′-TGCAGTATGAGTTGAATCGG-3′ Rv: 5′-AGGGAGGGGAGCCGGCTGTC-3′	30 cycles: 94°C 30 s, 60°C 1 min, 72°C 1 min	740	Gene Bank an J03572, M19070. M1970
OPN	Fw 5′-TCACCATTCCGATGAATCTG-3′ Rv 5′-ACTCGTGGCTCTGATGTTCC-3′	30 cycles: 94°C 30 s, 51°C 30 s, 72°C 30 s	505	Gene Bank An M14656
OCN	Fw 5′-GACCCTCTCTCTGCTCAC-3′ Rv 5′-GTGGTCCGCTAGCTCGTC-3′	25 cycles: 94°C 45 s, 55°C 45 s, 72°C 45 s	250	Gene Bank An NM0134114
BMP-2	Fw 5′-GAGTTTGAGTTGAGGCTGCTC-3′ Rv 5′-TGAGTCACTAACCTGGTGTCC-3′	35 cycles: 94°C 1 min, 58°C 1 min, 72°C 1 min	440	Bodamyali et al., 1998
BMP-4	Fw 5′-AGGCGCGACAGATGCTAGTT-3′ Rv 5′-GTCCAGGCACCATTTCTGCT-3′	35 cycles: 95°C1 min, 55°C 1 min, 72°C 1 min	526	Gene Bank An Z22607
IL-6	Fw 5′-CACTAGGTTTGCCGAGTAGATCTC-3′ Rv 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′	35 cycles: 95°C 1 min, 65°C 1 min, 72°C 1 min	638	Gene Bank An J03783
IL-1β	Fw 5′-ATGGCAACTGTTCCTGAACTCAAGT-3′ Rv 5′-CAGGACAGGTATAGATTCTTTCCTTT-3′	30 cycles: 95°C 1 min, 55°C 1 min, 72°C 1 min	563	Gene Bank An NM008361
GAPDH	Fw 5′-CTGCACCACCAACTGCTTAG-3′ Rv 5′-AGATCCACAACGGATACATT-3′	25 cycles: 94°C 30 s, 51°C 30 s, 72°C 30 s	282	Gene Bank An AF106860

Bp, base pairs; Fw, forward; Rv, reverse; an, accession number.

DNA Fragmentation Assay

Cells were lysed in 100 μl of lysis buffer (10 mM Tris HCl, pH 8, 25 mM EDTA, 0.5% SDS) for 4 h at 56°C and centrifuged at 12,000 rpm. Supernatant was recovered and DNA precipitated with sodium acetate and ethanol. After centrifugation at 12,000 rpm, the pellet was resuspended in Tris-EDTA buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and electrophoresed in 2% agarose gel containing ethidium bromide.

Densitometric Analysis

Scanning densitometry of the band areas was performed using the Molecular Analyst software for the model 670 scanning densitometer of the Bio-Rad Laboratories (Hercules, CA). Normalization for immunoblotting and RT-PCR was performed using as internal controls the reference genes β-actin and GAPDH, respectively. Density ratio between each gene of interest and the internal control was then computed and represented in graphs as arbitrary density units.

Statistics

Data are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was performed by the unpaired Student's *t*-test. A P value < 0.05 was conventionally considered statistically significant.

RESULTS

ROS.SMER#14 Morphology

As expected [Zhau et al., 1997], phase contrast microscopy showed that ROS.SMER#14 cells cultured in RWV formed multicellular aggregates termed organoids. These organoids comprised a variable number of cells and were formed only when vessels were rotating in RWV. Organoid formation was observed within 48 h of culture under rotation. In N-RWV, cell aggregates did not form and cells remained in non-attached conditions. In tissue culture dishes, cells attached to substratum and formed typical multilayer nodules (Fig. 2).

ROS.SMER#14 Cell Phenotype

In RWV culture condition, ROS.SMER#14 cells maintained all the osteoblast markers. In comparison to conventional tissue cultures, ALP activity and mRNA expression were incremented. In contrast, these markers resulted diminished in cells grown in N-RWV (Fig. 3). The expression of osteopontin (OPN), a sialoprotein of bone synthesized by differentiated osteoblasts, was increased both at the mRNA and protein levels (Fig. 4). Interestingly, the protein showed two bands, of which that at the highest molecular weight was assumed to be highly glycosilated, sulfated or phosphorylated [Singh et al., 1990]. This band was abundantly upregulated when cells were cultured in RWV. A modest increase of this band was instead observed in cells cultured in suspension in N-RWV (Fig. 4B). We also analyzed the expression of osteocalcin (OCN) by RT-PCR which showed a significant increase of mRNA under microgravity conditions compared to both controls (Fig. 5). Two other proteins of bone, the bone sialoprotein II (BSP-II) and the osteonectin (ON), were expressed by ROS.SMER#14 cells. However, their expression was not significantly changed

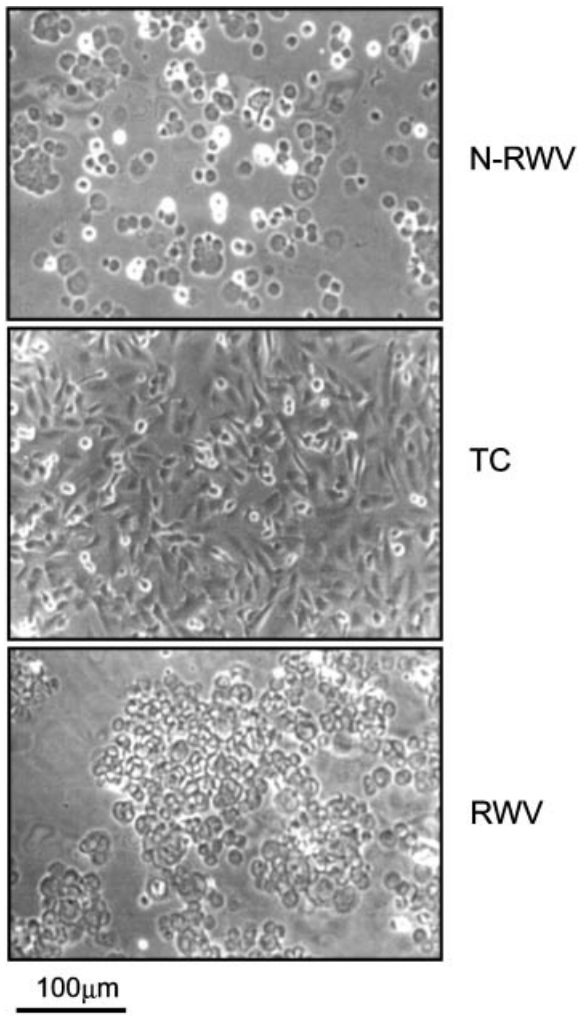

Fig. 2. Osteoblast morphology. ROS.SMER#14 cells were cultured in tissue culture dishes (TC) in N-RWV at unit gravity and in the RWV subjected to a rotation of 16 rpm. After 48 h, cells were fixed in 3% paraformaldehyde and observed by phase contrast microscopy. Original magnification 200×.

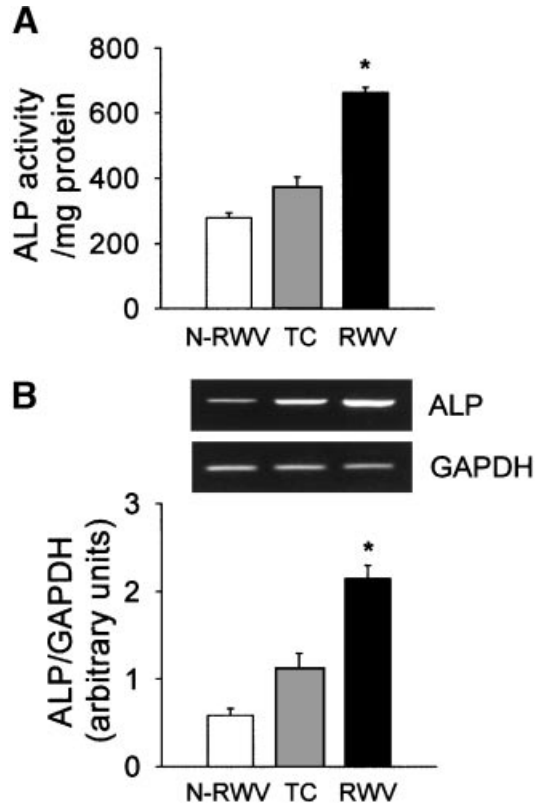

Fig. 3. Alkaline phosphatase (ALP) activity and mRNA expression. ROS.SMER#14 cells were cultured for 48 h in tissue culture dishes (TC) in N-RWV at unit gravity and in the RWV subjected to a rotation of 16 rpm (microgravity at 0.008*g*). **A**: Biochemical assay. Cells were lysed in 0.1% SDS and ALP activity was evaluated biochemically using *p*-nitrophenyl-phosphate as substrate (Sigma kit no. 104). Data represent mean ± SEM of three independent experiments (*$P < 0.005$ vs. N-RWV and TC). **B**: RT-PCR. Cells were collected and RNA extracted. One microgram of RNA was reverse-transcribed and the equivalent of 0.1 μg subjected to PCR using primer pairs and conditions as described in Table I. Densitometric analysis was performed and values obtained were expressed as ratio between ALP and the house-keeping gene GAPDH. Similar results were observed in three independent experiments (*$P < 0.05$ vs. N-RWV and TC).

in cells cultured both in N-RWV and in RWV compared to cells grown in tissue culture (Fig. 6).

We next analyzed the expression of mRNA for two major regulators of bone function, the bone morphogenetic proteins (BMP)-2 and 4. Figure 7 shows that BMP-2 was expressed at equal levels in every culture condition. In contrast, the BMP-4 mRNA was upregulated in cells grown in RWV compared to cells grown in conventional tissue culture or in N-RWV.

Osteoblast Proliferation and Viability

Since the reduced gravity forces are known to reduce osteoblast function, which results in severe bone loss, we found the previous results surprising. Therefore, we were prompted to explore the possibility that other factors could contribute to reduced osteogenic function of osteoblasts subjected to microgravity in RWV. To this purpose, we first analyzed changes in cell number and viability. A significant reduction of total cell number was indeed observed both in RWV and in N-RWV relative to tissue culture conditions (Fig. 8). In addition, according to the trypan blue exclusion test, a portion of these cells was nonviable (percent of death cells ± SEM: N-RWV = 35.75 ± 1.75; TC = 5.8 ± 3; RWV = 14.2 ± 7).

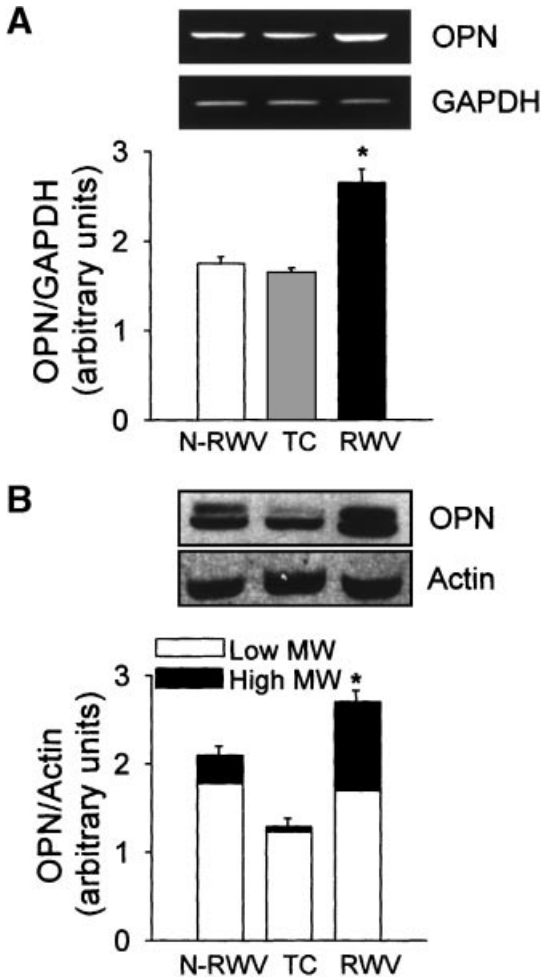

Fig. 4. Osteopontin (OPN) expression. ROS.SMER#14 cells were cultured for 48 h as described in Figure 3. **A**: RT-PCR. Cells were collected and RNA extracted. 1 μg of RNA was reverse-transcribed and the equivalent of 0.1 μg subjected to PCR using primer pairs and conditions as described in Table I. The values obtained by densitometric analysis were expressed as ratio between OPN and the house-keeping gene GAPDH. Similar results were obtained in three independent experiments (*$P < 0.05$ vs. N-RWV and TC). **B**: Western blotting. Cells were lysed in RIPA buffer and 25 μg of proteins were subjected to 10% SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-OPN polyclonal antobody (diluted 1:1,000), followed by incubation with HRP-conjugated secondary antibody (diluted 1:5,000). Filter was stripped and reprobed with anti-actin polyclonal antibody (diluted 1:500). Bands were detected by ECL and evaluated by densitometric analysis, then the density ratio between OPN and the constitutive protein actin was computed. Similar results were obtained in three independent experiments with a $P < 0.05$ vs. N-RWV and TC.

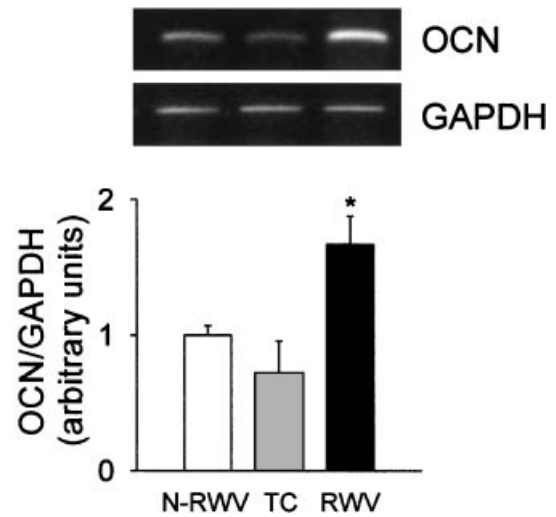

Fig. 5. mRNA regulation of Osteocalcin (OCN). ROS.SMER#14 cells were cultured for 48 h as described in Figure 3. RNA was extracted, 1μg was reverse-transcribed and the equivalent of 0.1 μg was subjected to PCR using the osteocalcin primer pairs and conditions as described in Table I. The values obtained by densitometric analysis of the bands were expressed as ratio between OCN and the house-keeping gene GAPDH. Similar results were obtained in three independent experiments with a $P < 0.05$ vs. N-RWV and TC.

To investigate whether cell damage was occurring, we first evaluated the overall morphology and cytoskeleton organization. To this purpose, cells were collected from each culture condition and attached to glass coverslips by cytospin procedure. Microfilament decoration by fluorescent phalloidin was then performed and cells observed by conventional epifluorescence. Figure 9 shows that cell aggregates of variable size were present in RWV. Cells were spherically shaped, with intense microfilament staining, and irregular edges. In contrast, cells harvested from tissue culture dishes were homogeneously flat and round, with regular edges and microfilaments forming a peripheral continuous ring. Cells grown in N-RWV, showed irregular size and shape, ranging from small spherical to large flat cells. Margins were irregular and the cytoskeleton was intensely stained and not well defined (Fig. 9). Cell fragments were also apparent. An estimation of cell size showed that the average diameter was similar in the three conditions, albeit with a larger standard error in RWV (13.4 ± 0.054, range 7.2–22.5 μm) and in N-RWV (12.6 ± 0.054, range 7.2–22.5 μm) compared to tissue culture dishes (12.6 ± 0.018, range 8.1–18.0 μm; $n = 3$ per each experimental point; n.s.)

In agreement with these morphological findings, DNA staining by bis-benzimide revealed regularly shaped nuclei in tissue culture dishes.

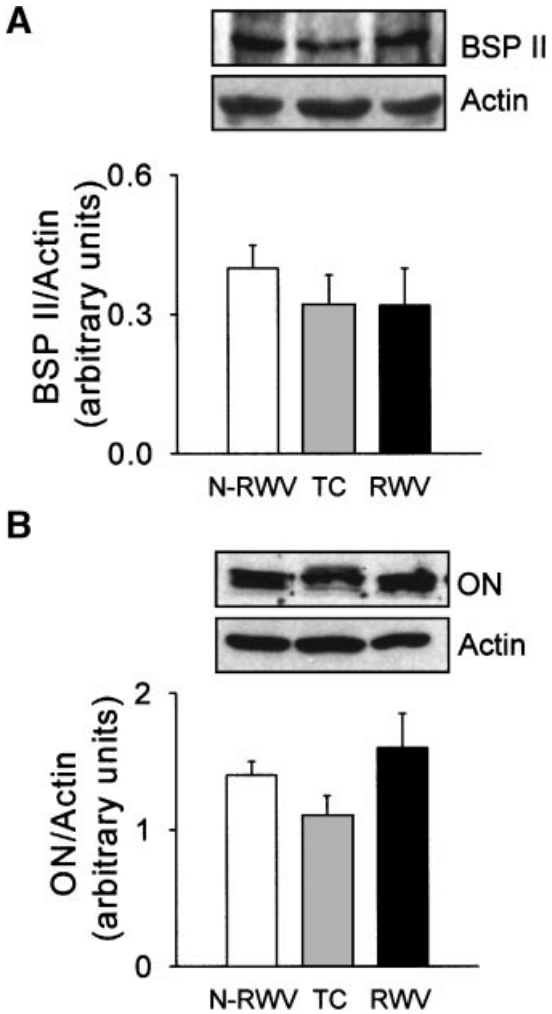

Fig. 6. Expression of bone sialoprotein II (BSP-II) and osteonectin (ON). ROS.SMER#14 cells were cultured for 48 h as described in Figure 3. Cells were lysed in RIPA buffer, and 25 μg of proteins were subjected to electrophoresis in a 10% SDS–PAGE. Filters were immunoblotted with anti BSP II polyclonal antibody, diluted 1:1,000 (**A**) and anti-ON polyclonal antibody, diluted 1:1,000 (**B**), followed by incubation with HRP-conjugated secondary antibody, diluted 1:5,000. Filters were then stripped and reprobed with anti-actin polyclonal antibody (diluted 1:500). Bands were detected and evaluated as described in Figure 4B. Similar results were obtained in three independent experiments. Differences were not statistically significant.

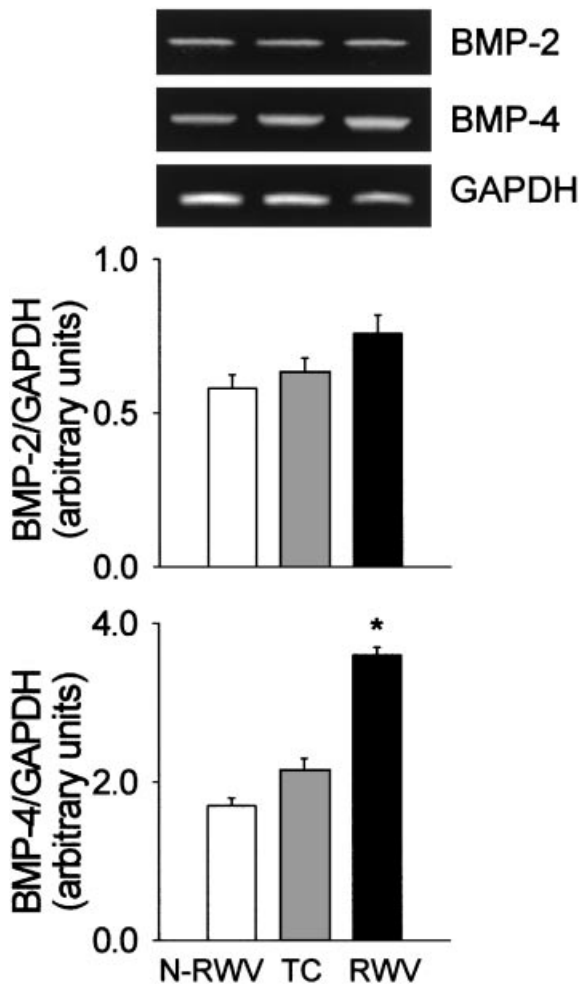

Fig. 7. mRNA regulation of bone morphogenetic proteins (BMP)-2 and 4. ROS.SMER#14 cells were cultured for 48 h as described in Figure 3. RNA was extracted, 1 μg was reverse-transcribed, and the equivalent of 0.1 μg was subjected to PCR using the BMP-2 and BMP-4 primer pairs and conditions as described in Table I. The values obtained by densitometric analysis of the bands were expressed as ratio between BMP-4 or BMP-2 and the house-keeping gene GAPDH. Similar results were obtained in three independent experiments with a $P < 0.05$ vs. N-RWV and TC for BMP-4 gene.

Nuclei were irregular both in RWV and in N-RWV, with a high degree of apoptotic appearance (Fig. 10A). This was confirmed by enumeration of nuclei with sign of DNA damage (Fig. 10B) and by electrophoretic analysis of nucleosome-sized DNA laddering (Fig. 10C). Despite this, immunoblotting analysis revealed no changes in the expression of a panel of genes implicated in the onset of apoptosis, i.e., p53, bcl-2, bax, and caspase 8 (Fig. 10D). In addition, p53 subcellular distribution was also found unmodified in apoptotic cultures versus non-apoptotic control (Fig. 10E).

Regulation of Cytokine mRNA

Osteoblasts are known to stimulate osteoclast differentiation and resorbing function by cell–cell interaction, and by the release of osteoclast regulating cytokines [Lowik et al., 1989; Manolagas, 1998]. Weightlessness stimulates osteoclast activity [Vico et al., 1987; Cavolina et al., 1997], therefore we examined the pattern of regulation of osteoclast-seeking cytokines in

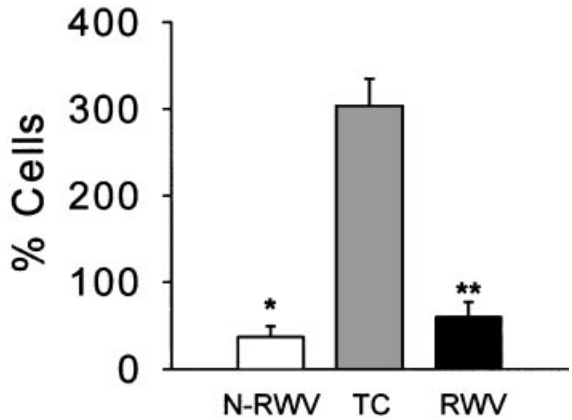

Fig. 8. Osteoblast number. ROS.SMER#14 cells were cultured at a density of 100,000/ml as described in Figure 3 for 48 h. At the end of incubation, cells were collected, counted under a phase contrast microscope, and expressed as a percentage relative to the initial number of cells inoculated in the culture vessels. Each point represents the mean ± SEM of data from three independent experiments (*$P < 0.003$ and **$P < 0.002$ vs. TC).

osteoblasts cultured in RWV. To accomplish this, we performed semiquantitative RT-PCR and observed that, unlike primary osteoblasts, ROS.SMER#14 cells did not express interleukin (IL)-1β in any culture condition. In contrast, these cells expressed IL-6. Compared to cells cultured in conventional tissue culture dishes and to cells grown in N-RWV, the expression of IL-6 mRNA appeared upregulated in RWV (Fig. 11). In our hands, this cytokine was able to stimulate osteoclast formation and bone resorption in mouse bone marrow cell cultures [Perez et al., 2001], thus revealing a potential role as a mediator of enhanced osteoclast activity under microgravity conditions.

Finally, we also analyzed the mRNA expression of OPG and OPGL, and found an increase of the OPGL/OPG rate in RWV compared to cells cultured in tissue culture conditions but not respect to cells grown in N-RWV (data not shown).

DISCUSSION

This study provided evidence that the RWV device allowed the culture of osteoblast-like cells, which maintained their phenotype, appeared more differentiated compared to conventional cultures at unit gravity but had a short life span likely due to enhancement of apoptosis.

Indeed, recent reports evaluated the ability to obtain three-dimensional osteoblast cell cultures using microcarriers in the RWV, thus simulating a condition of microgravity. In particular, cultures of bone marrow stromal cells or ROS 17/2.8 cells in the RWV showed that they attached to and formed three-dimensional aggregates with the microspheres [Qiu et al., 1999, 2001]. Moreover, extracellular matrix and

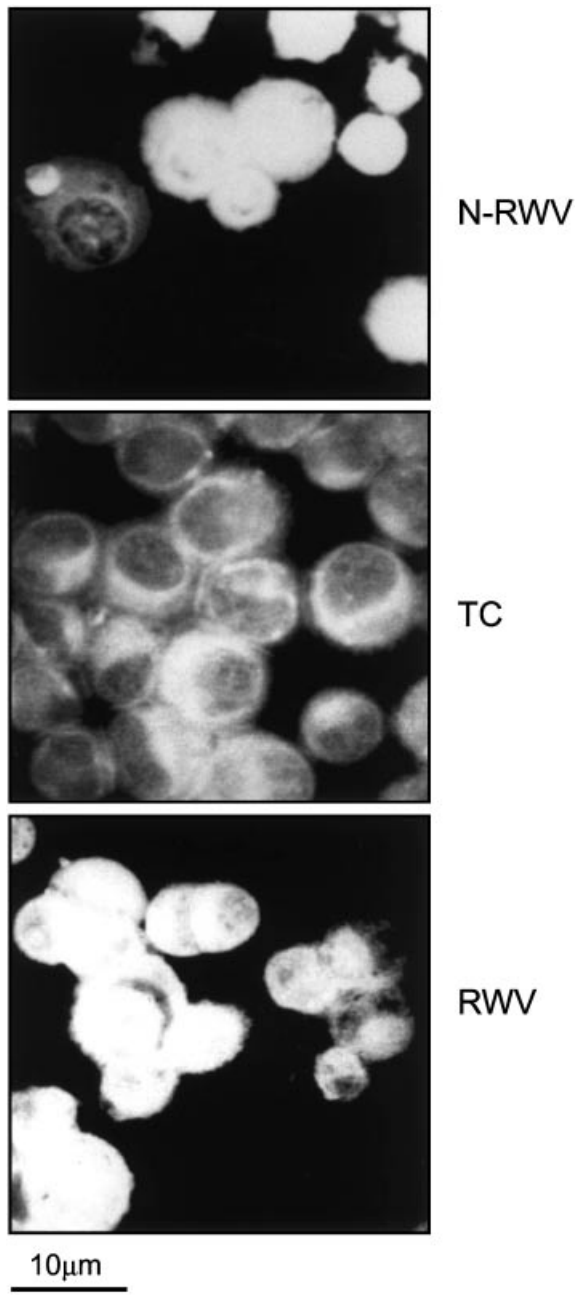

Fig. 9. Osteoblast morphology and cytoskeleton organization. ROS.SMER#14 cells were cultured for 48 h as described in Figure 3, then attached to glass slides by cytospin procedure, fixed, stained with rodhamine thiocyanate-conjugated phalloidin and observed by conventional epifluorescence. Original magnification 630×.

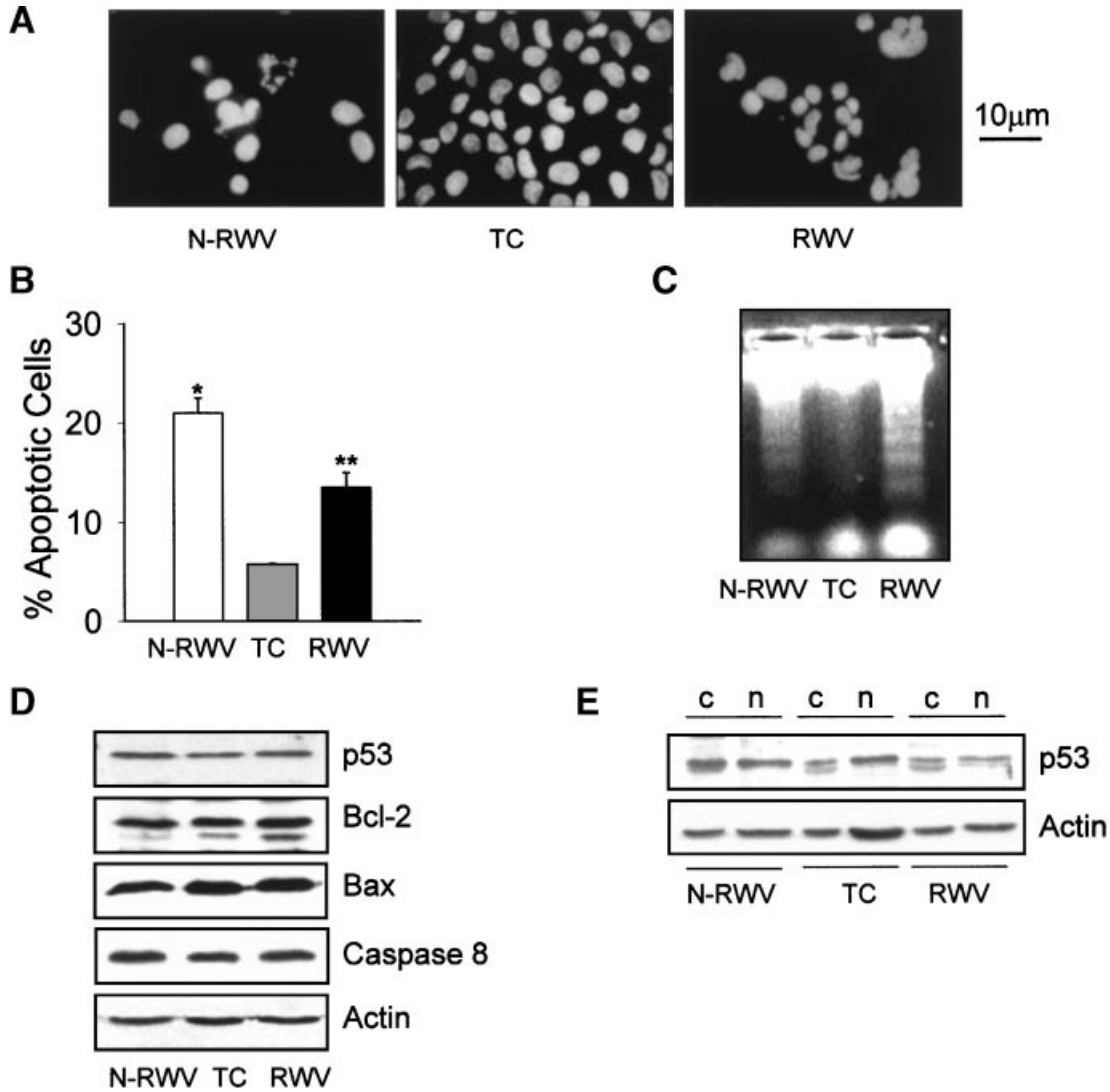

Fig. 10. Osteoblast apoptosis. ROS.SMER#14 cells were cultured for 48 h as described in Figure 3. **A**: Cells from each culture condition were attached to glass slides by cytospin procedure, fixed, and stained with bis-benzimide. Nuclear morphology was then evaluated by conventional epifluorescence microscopy. Pictures are representative of three independent experiments. Original magnification 630×. **B**: The total number of cells present in at least five random microscopic fields was counted and the percentage of cells with apoptotic nuclei was computed. Data represent the mean ± SEM of three independent experiments (*$P < 0.005$ and **$P < 0.006$ vs. TC). **C**: Cells were lysed as described in Materials and Methods, and the DNA was subjected to electrophoresis in a 2% agarose gel containing ethidium bromide. **D**: Cells were lysed in RIPA buffer, then 25 μg of proteins were electrophoresed in 12% SDS–PAGE and blotted to nitrocellulose membranes. Filters were respectively probed with p53 (diluted 1:500), Bax (diluted 1:500), Bcl-2 (diluted 1:500), caspase 8 (diluted 1:500) polyclonal antibodies, followed by incubation with HRP-conjugated secondary antibody (diluted 1:5,000). Membranes were also stripped and reprobed with anti-actin polyclonal antibody (diluted 1:500). Bands were detected and evaluated as described in Figure 4. **E**: Cytosolic (c) and nuclear (n) proteins were fractionated as described in Materials and Methods, then 25 μg were electrophoresed in 12% SDS–PAGE and analyzed for p53 distribution as described in (D). Results described in C–E are representative of three independent experiments each.

mineralization were also observed [Qiu et al., 1999].

Microgravity conditions obtained in the RWV [Duray et al., 1997; Freed et al., 1997] are similar to those experienced in parabolic flights by aircraft [Schwarz et al., 1992]. With respect to parabolic flights, however, the RWV offers the possibility of longer-term experiments, a significant advantage if we take into account the fact that tissue homeostasis is altered by exposition to weightlessness for days [Turner, 2000]. One of the pitfalls of RWV is the lack of

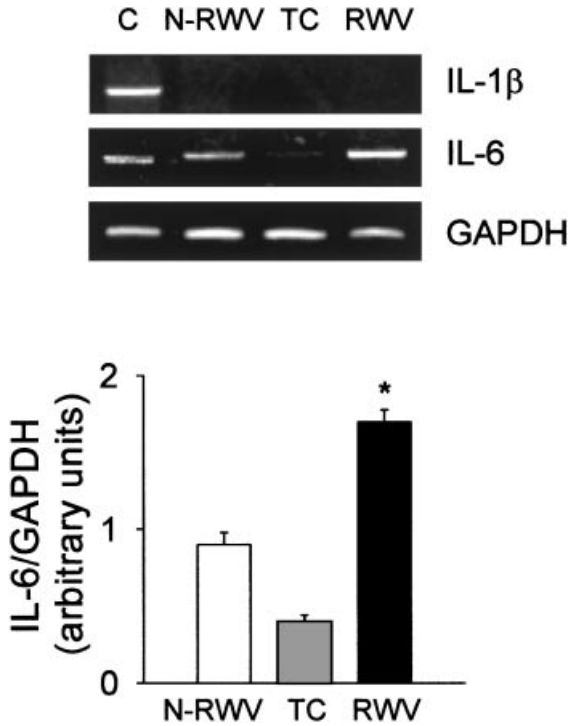

Fig. 11. mRNA regulation of IL-1β and IL-6. ROS.SMER#14 cells were cultured for 48 h as described in Figure 3. RNA was extracted, 1 μg was reverse-transcribed, and the equivalent of 0.1 μg was subjected to PCR using the IL-1β and IL-6 primer pairs and conditions as described in Table I. RNA extracted from a rat primary osteoblast culture was used as positive control (C). For IL-6, the values obtained by densitometric analysis of the bands were expressed as ratio between IL-6 and the housekeeping gene GAPDH. Similar results were obtained in three independent experiments with a $P < 0.05$ between RWV and TC.

perfectly matched controls. Our investigation was focused on the effect of microgravity, obtained with this particular method, on osteoblast phenotype. For comparison, we used two other culture conditions. The conventional tissue culture dishes maintained our cells at unit gravity and allowed cell–cell and cell–matrix interactions. Although osteoblasts form nodules, this condition cannot be assimilated to the formation of organoids as those observed in RWV. Alternatively, we used as a control the same vessel of the bioreactor not subjected to rotation. In this condition, cells are maintained at unit gravity, however, they do not attach to a substratum and their cell–cell and cell–matrix relationships are likely to be altered. According to in vivo data obtained in astronauts and animal models, 1–2% of bone loss occurs during space-flights over a period of 30 days, and this effect is believed to depend on reduced bone formation in the face of unaltered or increased bone resorption [Caillot-Augusseau et al., 2000]. Therefore, it is expected that osteoblast differentiation, maturation, and bone forming activity are impaired in weightlessness conditions [Carmeliet and Bouillon, 1999]. This has been demonstrated in a study [Landis et al., 2000] showing that osteoblasts subjected to space-flights followed a slower progression toward a differentiated function. In contrast, Harris et al. [2000] showed no changes in ALP activity and collagen type I expression in human osteoblasts subjected to space-flights for 17 days. Others have shown that longitudinal bone growth is not affected in space-flights and in simulated weightlessness conditions [Sibonga et al., 2000].

In the cell model presented in this study, the ROS.SMER#14 cell line, we showed a remarkable stimulation of some osteoblast markers at least compared to our controls. These results are in part similar to those observed by Granet et al. [1998] who observed that ROS 17/2.8 cultured in the RWV had the same rate of proliferation with respect to both controls, but appeared more differentiated after 10 days of culture. In contrast, Kunisada et al. [1997] showed a reduction of ALP activity and osteocalcin production under microgravity conditions. We believe that one explanation of our data is that the reduced gravity could activate the expression of osteoblast differentiating genes to compensate the effect of negative loading. Moreover, our results could be due to the peculiar culture conditions in which the osteoblasts can interact among them in a close physiologic environment, as that created by the organoids. According to this possibility, it has been shown that tissues maintained in RWV in organ cultures keep their overall structure unaltered compared to the original explants [Lewis et al., 1993; Hatfill et al., 1996].

Strikingly, our investigation shows that despite the maintenance of a differentiated phenotype, ROS.SMER#14 cells cultured in RWV under microgravity suffer of reduced proliferation and viability, the latter likely determined by enhanced apoptosis. This event, as also suggested by Sarkar et al. [2000] could, therefore, significantly reduce the size of the osteoblast population, leading to the long-term effect shown in other studies, i.e., reduced bone formation. Unfortunately, we were unable to dissect the molecular signaling triggering apoptosis. The p53 pro-apoptotic transcription factor was not enhanced nor its subcellular distribution

was modified. Similarly, the bcl-2/bax ratio, whose unbalance in favor of bax leads to cytochrome c release from mitochondria and subsequent activation of the caspase cascade [Green, 2000], was not altered under microgravity. In agreement with this findings, the caspase 8, a leading pro-apoptotic enzyme, was not cleaved and therefore remained inactive [Hengartner, 2000]. Therefore, at present we cannot draw conclusions on the intracellular signals to apoptosis triggered in ROS.SMER#14 cells in our experimental model. It must be pointed out that cells cultured in N-RWV also showed reduced viability, an effect that we hypothesized to depend on the incapability of these cells to attach each other or to the substratum, a condition that is known to trigger apoptosis [Rodeck et al., 1997]. Whether or not the altered viability and apoptosis in RWV are due to the same mechanisms activated in N-RWV remains to be elucidated.

Bone homeostasis is due to the concerted activity of osteoblasts and osteoclasts. Osteoblasts build the bone and osteoclasts destroy it, and the harmonic activity of the two cell types leads to a balance that allows the skeleton to maintain its mass and contribute to extracellular fluid ion equilibrium throughout our life. There are two ways by which an individual can lose bone: by impaired osteoblast bone formation and by enhanced osteoclast bone resorption [Rodan, 1998]. In fact, an unbalance between the activity of the two cell types is known to lead to osteopenic syndromes, first of all the osteoporosis [Manolagas, 2000]. Osteoblasts not only form bone but also influence osteoclast activity. For instance, it has been established that the Osteoprotegerin ligand (OPGL) (also known as RANKL, TRANCE, and ODF) expressed at the osteoblast plasma membrane interacts with the OPGL receptor, known as RANK, expressed on osteoclasts and osteoclast precursors. This interaction triggers a cascade of events leading to activation of the NF-κB transcription factor which, in turn, determines osteoclast differentiation and enhances mature osteoclast function [Lacey et al., 1998; Burgess et al., 1999]. Osteoblast paracrine stimuli are added to the bone microenvironment and further stimulate osteoclast function. Among these, the IL-1β and the IL-6 are considered major inductors of bone loss, for instance in post-menopausal osteoporosis [Manolagas, 1998]. In our hands, ROS.SMER#14 cells failed to express IL-1β, and this remained unmodified in each culture condition. In contrast, the IL-6 was barely expressed in conventional tissue cultures but resulted significantly enhanced in RWV. We have recently demonstrated that IL-6 stimulates osteoclast formation and enhances bone resorption in mouse bone marrow cultures [Perez et al., 2001], providing evidence that IL-6 actually positively affects osteoclast activity. Therefore, it is worthy to conclude that one means by which osteoblasts could contribute to bone loss under microgravity conditions is associated with the paracrine enhancement of osteoclast-mediated bone resorption, for instance through the IL-6 pathway. These events occurring in bone, associated with alterations of other structures, such as the muscles and the blood vessels, could contribute to the typical osteopenic syndromes casued by mechanical unloading.

In summary, this study provides a new tool to investigate the homeostasis of osteoblasts subjected to simulated microgravity on ground, and strikingly suggest that reduced cell viability and enhancement of IL-6 may represent important mechanisms underlying bone loss in individuals subjected to weightlessness. Due to the large heterogeneity and often contradictory findings reported on this issue [Marie et al., 2000; Turner, 2000], at the moment it is not safe to draw conclusions on the molecular mechanisms whereby the skeleton of astronauts is negatively affected by weightless. However, we believe that this new tool will allow in the next future to obtain further insights into this important aspect of bone cell biology.

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