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Modeled Microgravity Stimulates Osteoclastogenesis and Bone Resorption by Increasing Osteoblast RANKL/OPG Ratio

Nadia Rucci, Anna Rufo, Marina Alamanou, and Anna Teti*

Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

Abstract Mechanical unloading causes detrimental effects on the skeleton, but the underlying mechanisms are still unclear. We investigated the effect of microgravity on osteoblast ability to regulate osteoclastogenesis. Mouse osteoblast primary cultures were grown for 24 h at unit gravity or under simulated microgravity, using the NASA-developed Rotating Wall Vessel bioreactor. Conditioned media (CM) from osteoblasts subjected to microgravity increased osteoclastogenesis and bone resorption in mouse bone marrow cultures. In these osteoblasts, the RANKL/OPG ratio was higher relative to 1*g*. Consistently, treatment with high concentrations of OPG-inhibited osteoclastogenesis and bone resorption in the presence of CM arising from osteoblasts cultured under microgravity. Microgravity failed to affect osteoblast differentiation and function in the time frame of the experiment, as we found no effect on *alkaline phosphatase* mRNA and activity, nor on *Runx2*, *osteocalcin*, *osteopontin*, and *collagen1A2* mRNA expression. In contrast, microgravity induced a time dependent increase of ERK-1/2 phosphorylation, while phospho-p38 and phospho-JNK remained unchanged. Apoptosis, revealed by bis-benzimide staining, was similar among the various gravity conditions, while it was increased under microgravity after treatment with the MEK-1/2 inhibitor, PD98059, suggesting a protection role by ERK-1/2 against cell death. In conclusion, microgravity is capable to indirectly stimulate osteoclast formation and activity by regulating osteoblast secretion of crucial regulatory factors such as RANKL and OPG. We hypothesize that this mechanism could contribute to bone loss in individuals subjected to weightlessness and other unloading conditions. J. Cell. Biochem. 100: 464–473, 2007.

Key words: microgravity; osteoclast; osteoblast; bone remodeling

Among the several physiological changes observed in humans exposed to weightlessness, skeletal alterations seem to be particularly serious, leading to bone loss and negative calcium balance [Caillot-Augusseau et al., 1998, 2000]. In the most severe conditions of weightlessness in humans, there is an approximately 2% decrease in bone mineral density in only 1 month [Collet et al., 1997]. This evidence is in agreement with the normal physiology of the bone tissue, that responds to mechanical loading by activation of bone formation and inhibition of bone resorption as demonstrated in rats [Forwood et al., 2001; Hsieh et al., 2001; Robling et al., 2001]. The principal effectors of bone mass regulation are the osteoclasts, which are the cells devoted to resorb bone, and the osteoblasts, with osteogenic functions. A perfect balance between the two functions accounts for the maintenance of a correct bone mass.

Ground-based studies performed using different models of simulated microgravity demonstrated that bone loss could be at least partially due to a decrease in bone formation by osteoblasts, whose differentiation and function are negatively affected. In particular, the gene expression of the osteoblast differentiation marker *alkaline phosphatase* (*Alp*), of *runt-related transcription factor2* (*Runx2*), and of the bone matrix proteins *osteocalcin* and *collagen I* are significantly decreased in murine preosteoblasts and human mesenchimal stem cells exposed to simulated microgravity [Zayzafoon et al., 2004; Pardo et al., 2005].

Nadia Rucci and Anna Rufo are contributed equally to this study.

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*Correspondence to: Anna Teti, PhD, Department of Experimental Medicine, Via Vetoio—Coppito 2, 67100 L'Aquila, Italy. E-mail: teti@univaq.it

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With regard to a potential effect of microgravity on bone resorption, an increase of bone resorption markers, such as urinary calcium levels and collagen cross-links, has been described in astronauts during 6 months space flight [Smith et al., 2005]. Histomorphometric data obtained from tibias of rats exposed for 7 days to weightlessness during a space flight, showed a markedly reduction of trabecular bone volume relative to control littermates, while resorption activity remained unchanged [Vico et al., 1988; Durnova et al., 1996]. These data have been refuted in a ground-based study performed in older rats, which showed that resorption was increased in unloading conditions, but formation was unchanged [Smith et al., 2002]. Conversely, in vitro studies aimed at evaluating the molecular mechanisms underlying bone loss, with particular regard to osteoclast differentiation and function, are still lacking.

The present study investigated the effect of modeled microgravity on ex vivo osteoblast-mediated osteoclastogenesis and bone resorption. To accomplish this, we used the NASA-developed Rotary Cell Culture System (RCCS) equipped with a Rotating Wall Vessel (RWV) bioreactor. The RCCS simulates microgravity by randomizing the gravitational vector in response to the rotation of the culture vessel around a horizontal axis [Rucci et al., 2002; Zayzafoon et al., 2004], with diffusion of oxygen and carbon dioxide occurring across a semi-permeable membrane. Our results demonstrated that conditioned media (CM) recovered from primary osteoblasts cultured for 24 h under 0.08 and 0.008*g* of gravity were able to significantly increase mouse bone marrow osteoclastogenesis and bone resorption relative to CM harvested from osteoblasts grown at unit gravity. An increase of the *Rankl*/*Opg* (*receptor activator of NFkB ligand*/*osteoprotegerin*) ratio was observed in osteoblasts subjected to modeled microgravity. This increase accounted for the osteoclastogenic effect of microgravity osteoblast CM, as demonstrated by the poor osteoclast formation rate observed in the presence of the RANKL decoy receptor, OPG.

MATERIALS AND METHODS

Materials

Dulbecco's modified minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin were from GIBCO (Uxbridge, UK). Sterile plastic ware was purchased from Falcon Becton-Dickinson (Cowley, Oxford, UK), or Costar (Cambridge, MA). The anti-phospho-p44/42 ERK-1/2 (#9106), -phospho stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (#9251), -phospho-p38 (#9211) antibodies, were from New England Biolabs, Inc. (Beverly, MA). The anti -ERK2 (#sc-154), -JNK (#sc-572), -p38 (#sc-728), and HRP-conjugated antibodies were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). The ECL kit was from Amersham Pharmacia Biotech (Milan, Italy). Primers and reagents for RT-PCR were from Invitrogen (Carlsbad, CA). ELISA kits for mouse soluble RANK-L (#MTR00) and mouse OPG (#MOP00) were purchased from R&D systems (Abingdon, UK). The Trizol reagent was from Invitrogen (Pisley, UK). The Brilliant® SYBR® Green QPCR master mix was purchased from Stratagene (La Jolla, CA). All the other reagents were of the purest grade from Sigma-Aldrich Co. (St. Louis, MO).

Rotating Wall Vessel Bioreactor (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. Cells and liquid culture media are placed in the space between the inner and the outer cylinders, and the assembled device is rotated about its longitudinal axis [Duray et al., 1997; Freed et al., 1997; Rucci et al., 2002].

Osteoblast Primary Cultures

Calvaria from 7-day-old CD1 mice were removed, cleaned free from soft tissues, and digested three times with 1 mg/ml *Chlostridium histolyticum* type IV collagenase and 0.25% trypsin, for 20 min at 37°C, with gentle agitation. Cells from the second and third digestion were plated and grown to confluence in DMEM plus 10% FBS. At confluence, cells were trypsinized by standard procedure and plated according to the experimental protocol. These cells expressed the osteoblast markers *alkaline phosphatase* (*Alp*), *runt-related transcription factor 2* (*Runx2*), *parathyroid hormone*/*parathyroid hormone-related peptide* (*PTH*/*PTHrP*) receptor, type I *collagen*, and *osteocalcin* (*Ocn*) [Marzia et al., 2000].

Differentiated osteoblasts were cultured for 24 h in RWV bioreactor vessels in three experimental conditions: no rotation, corresponding to a condition of unit gravity (1*g*), 50 rpm rotation and 16 rpm rotation which lead to microgravity conditions of 0.08 and 0.008*g*, respectively. Each experiment was repeated at least three times with different primary osteoblast preparations.

Osteoclast Primary Cultures

Primary osteoclasts were differentiated from the bone marrow of 7-day-old CD1 mice. Bone marrow was flushed from the bone cavity of the long bones and minced in DMEM. Cells were recovered, plated in DMEM plus 10% FBS, and cultured up to 7 days in the presence of 1, 0.08, and 0.008*g* osteoblast-CM to assess osteoclast differentiation by paracrine osteoblast factors. Cultures were fixed in 3% paraformaldehyde in 0.1 M cacodylate buffer, and positive staining for the osteoclast marker enzyme tartrate resistant acid phosphatase (TRAcP) was detected histochemically using the Sigma-Aldrich kit No. 85, according to the manufacturer's instructions.

Osteoclasts were also differentiated as described onto bone slices and fixed in 3% paraformaldehyde in 0.1 M cacodylate buffer. Cells were then removed by ultrasonication in 1% sodium hypochlorite, and slices were stained with 0.1% toluidine blue. Pits were counted and the pit index computed according to Caselli et al. [1997]. Both osteoclastogenesis and pit assays were repeated at least three times with independent cell preparations.

Western Blotting

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. Proteins were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes. Blots were probed with the primary antibody for the indicated phosphorylated mitogen-activated protein kinases (MAPKs), for 1 h at room temperature, washed and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. Filters were then stripped by incubation with the stripping solution (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) for 30 min at 50°C and reprobed as described above to detect total MAPKs as loading control. Protein bands were revealed by enhanced chemiluminescence (ECL).

RT-PCR and Real Time PCR

Total RNA was extracted using the Trizol® procedure. 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. For real time PCR, the Brilliant® SYBR® Green QPCR master mix was used. PCR conditions and primer pairs used are listed in Table I.

Determination of Soluble RANK-L and OPG in Conditioned Media by ELISA.

Soluble RANK-L and OPG were quantified in CM using the R&D System ELISA kits #MTR00 and #MOP00, respectively, according to the manufacturer's instructions.

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 instruction.

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 bis-benzimide, rinsed twice in distilled water, mounted in glycerol-PBS 1:1, and observed by conventional epifluorescence microscopy.

Statistics

Data are expressed as the mean ± SD of at least three independent experiments each performed in quadruplicate wells. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the unpaired Student's *t*-test. A P value <0.05 was conventionally considered statistically significant.

RESULTS

Effect of Microgravity on Osteoclastogenesis and Bone Resorption

It is well known that osteoblasts play a crucial role in osteoclast differentiation and function through the synthesis of paracrine factors.

TABLE I. Primer Pairs and PCR Conditions

Gene	Primers	PCR conditions
Gapdh	Fw 5′-CACCATGGAGAAGGCCGGGG-3′ Rv 5′-GACGGACACATTGGGGGTAG-3′	25 cycles: 94°C 1 min, 55°C 1 min, 72°C 1 min
Alp	Fw 5′-CCAGCAGGTTTCTCTCTTGG-3′ Rv 5′-CTGGGAGTCTCATCCTGAGC-3′	35 cycles: 94°C 1 min, 60°C 1 min, 72°C 1 min
Opn	Fw 5′-CTTGCTTGGGTTTGCAGTCT-3′ Rv 5′-GCCAAATAGGCAAAAGCAAA-3′	35 cycles: 94°C 1 min, 60°C 1 min, 72°C 1 min
Ocn	Fw 5′-ACCCTGGCTGCGCTCTGTCTCT-3′ Rv 5′-GATGCGTTTGTAGGCGGTCTTCA-3′	35 cycles: 94°C 45 s, 60°C 45 s, 72°C 45 s
Col1A2	Fw 5′-GCAATCGGGATCAGTACGAA-3′ Rv 5′-CTTTCACGCCTTTGAAGCGA-3′	30 cycles: 94°C 1 min, 57°C 2 min, 72°C 1 min
Runx2	Fw 5′-AACCCACGGCCCTCCCTGAACTCT-3′ Rv 5′-ACTGGCGGGGTGTAGGTAAAGGTG-3′	35 cycles: 94°C 45 s, 60°C 45 s, 72°C 45 s
IL-6	Fw 5′-GAGGATACCACTCCCAACAGACC-3′ Rv 5′-AAGTGCATCATCGTTGTTCATACA-3′	40 cycles: 94°C 45 s, 60°C 45 s, 72°C 45 s
IL1-β	Fw 5′-CAACCAACAAGTGATATTCTCCATG-3′ Rv 5′-GATCCACACTCTCCAGCTGCA-3′	40 cycles: 94°C 45 s, 60°C 45 s, 72°C 45 s
PTHrP	Fw 5′-TGGTGTTCCTGCTCAGCTA-3′ Rv 5′-CCTCGTCGTCTGACCCAAA-3′	35 cycles: 95°C 1 min, 55°C 1 min, 72°C 1 min
M-CSF	Fw 5′-GAATCTTCACTGGGCACTAAC-3′ Rv 5′-CTTCCCATATGTCTCCTTTCC-3′	30 cycles: 94°C 40 s, 50°C 45 s, 72°C 1 min
TNF-α	Fw 5′-GCAGGTCTACTTTGGAGTCATTGC-3′ Rv 5′-TCCCTTTGCAGAACTCAGGAATGG-3′	30 cycles: 94°C 15 s, 58°C 15 s, 72°C 30 s
Opg	Fw 5′-AGTCCGTGAAGCAGGAGT-3′ Rv 5′-CCATCTGGACATTTTTTGCAAA-3′	40 cycles: 94°C 45 s, 55°C 45 s, 72°C 45 s
Rankl	Fw 5′-CCAAGATCTCTAACATGACG-3′ Rv 5′-CACCATCAGCTGAAGATAGT-3′	40 cycles: 94°C 45 s, 58°C 45 s, 72°C 45 s
GM-CSF	Fw 5′-ATGTGGCTGCAGAATTTACT-3′ Rv 5′-TTGTGTTTCACAGTCCGTTTCC-3′	35 cycles: 94°C 1 min, 58°C 30 s, 72°C 30 s
IL-12	Fw 5′-TTCAACATCAAGAGCAGTAG-3′ Rv 5′-AGAACTTGAGGGAGAAGTAG-3′	35 cycles: 94°C 30 s, 58°C 30 s, 72°C 30 s
IL-18	Fw 5′-CATCATCTTCCTTTTGGCAA-3′ Rv 5′-ACTGTACAACCGCAGTAATACGG-3′	30 cycles: 94°C 1 min, 52°C 1 min, 72°C 1 min

Based on this notion, we incubated mouse bone marrow cultures with CM from osteoblasts harvested after 24 h of culture at 1, 0.08, and 0.008*g*. We found a progressive increase of osteoclastogenesis (Fig. 1A) and bone resorption (Fig. 1B) after treatment with CM from osteoblasts grown under microgravity relative to unit gravity condition, with a maximum effect at 0.008*g*.

In order to dissect the underlying molecular pathways, we evaluated mRNA expression of cytokines regulating osteoclastogenesis in osteoblasts cultured in the three experimental conditions. Among the factors analyzed, we found similar levels of *interleukin-6* (*IL-6*), *interleukin1β* (*IL-1β*), *PTHrP*, and *tumor necrosis factor α* (*TNF-α*) (Fig. 2Aa) and of factors inhibiting osteoclast differentiation, such as *IL-12*, *IL-18*, and *GM-CSF* (*granulocyte monocyte-colony stimulating factor*) (Fig. 2Ab). In contrast, *Rankl* was progressively increased at 0.08 and 0.008*g* as detected by conventional (not shown) and comparative real time RT-PCR (relative expression ± SD: 4.2 ± 0.45 for 0.08*g* and 5.0 ± 0.3 for 0.008*g* vs. 1*g*, Fig. 2Ba). Moreover *Opg*, which binds RANKL blocking its interaction with the RANK receptor, was decreased (relative expression ± SD: 0.65 ± 0.05 for 0.08*g* and 0.54 ± 0.08 vs. 1*g*, Fig. 2Bb). These modulations led to a significant and progressive increase of the *Rankl*/*Opg* ratio in osteoblasts grown at 0.08*g* (6.4 ± 0.2-fold increase) and 0.008*g* (9.3 ± 2.0-fold increase) relative to 1*g* condition (Fig. 2Bc). Importantly, ELISA assays demonstrated that microgravity induced a modest but progressive increase of soluble RANKL (Fig. 2Ca) and a strong decrease of OPG (Fig. 2Cb) released in the culture media, with the final result of a potent increase of the RANKL/OPG ratio also at the level of secreted proteins (0.26 ± 0.08 for 0.08*g* and 0.76 ± 0.14 for 0.008*g* vs. 0.185 ± 0.14 for 1*g*) (Fig. 2Cc). Remarkably, the increment of soluble RANKL in the culture media is not as high as the increment of *Rankl* mRNA, possibly depending on most of the transcript being translated into the membrane-bound form of the cytokine.

To confirm a RANKL-mediated effect on microgravity-induced osteoclastogenesis and bone resorption, mouse bone marrow cells were cultured in the presence of osteoblast CM with or without the addition of excess of OPG. OPG treatment significantly inhibited osteoclast formation induced by the CM of osteoblasts

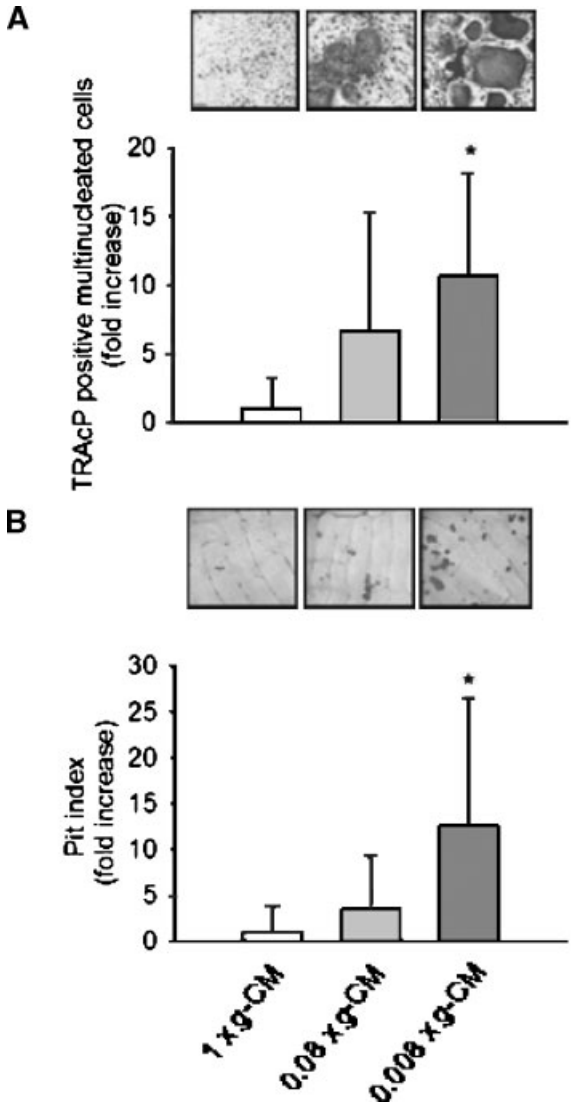

Fig. 1. Effect of modeled microgravity on osteoclastogenesis and bone resorption. **A**: The bone marrow from long bones of 7-day-old mice was cultured onto 24-well multiplates in the presence of serum-free conditioned media (CM) from osteoblasts grown for 24 h at 1, at 0.08, and at 0.008*g* of microgravity, using the NASA-developed RWV bioreactor. After 7 days, cultures were fixed and differentiated osteoclasts were stained for the tartrate-resistant acid phosphatase (TRAcP) enzyme (**upper panels**) and counted. Original magnification 100×. Data are expressed as mean ± SD of three independent experiments (*$P = 0.012$ vs. 1*g* CM). **B**: Bone marrow was cultured as described in (A) onto bone slices. After 7 days, slices were fixed and stained with toluidine blue (upper panels) and the pit index was calculated. Original magnification 100×. Data are expressed as mean ± SD of three independent experiments (*$P = 0.035$ vs. 1*g* CM).

cultured at 0.08 and 0.008*g*, returning osteoclastogenesis to levels comparable to the control condition (1*g*) (Fig. 3A). A similar pattern of inhibition was found for bone resorption (Fig. 3B).

Osteoblast Phenotype and Function

We next investigated if, in our short-term conditions, microgravity could affect osteoblast function and differentiation by evaluating osteoblast markers and expression of bone matrix proteins.

As showed in Figure 4, *Alp* mRNA expression (Fig. 4A) and activity (Fig. 4B) were similar in all the culture conditions. Moreover, *Runx2* transcription factor, its downstream gene *osteocalcin* (*Ocn*), the sialoprotein *osteopontin* (*Opn*), and *collagen 1A2* chain (*Col1A2*) mRNA were also unaffected by microgravity (Fig. 4A).

MAPKs Activation by Microgravity

To establish the mechanisms whereby microgravity signals the osteoblasts, we evaluated the phosphorylation status of kinases known for their central role in osteoblast activity. While c-Src and members of the protein kinase C (PKC) subclasses (PKCalpha, beta, delta, epsilon, and zeta) were unremarkable (not shown), MAPKs showed selective modulation. As showed in Figure 5 (upper middle panels), at 0.08*g* we found an increase of ERK-1/2 phosphorylation starting at 60 min of exposure to microgravity, which was maintained at 10 and 24 h. Activation of ERK-1/2 was even higher and occurred earlier at 0.008*g*, starting at 30 min and reaching a higher plateau at 10 h (Fig. 5, upper right panels). Phospho-p38 was time-dependently increased in all the experimental conditions (Fig. 5, middle panels) suggesting no dependence of its activation upon microgravity, while phospho-JNK was undetectable in any of the cultures (Fig. 5, lower panels).

Because ERK activation is known to prevent apoptosis [Almeida et al., 2005] we incubated for 24 h osteoblasts at 1*g* and under microgravity in the presence of the PD98059 inhibitor which blocks the ERK-1/2 activating kinase, MEK-1/2. This treatment prevented the increase of ERK-1/2 phosphorylation under microgravity (Fig. 6A). DNA staining by bisbenzimide showed a similar number of apoptotic nuclei at 1*g* as well as at 0.08 and 0.008*g* (Fig. 6B). However, at 0.08*g* and even more at 0.008*g* prevention of ERK-1/2 phosphorylation by PD98059 increased apoptosis by 1.4- and 1.5-fold, respectively (Fig. 6B), suggesting that some protection by phospho-ERK-1/2 against apoptosis under microgravity is conceivable.

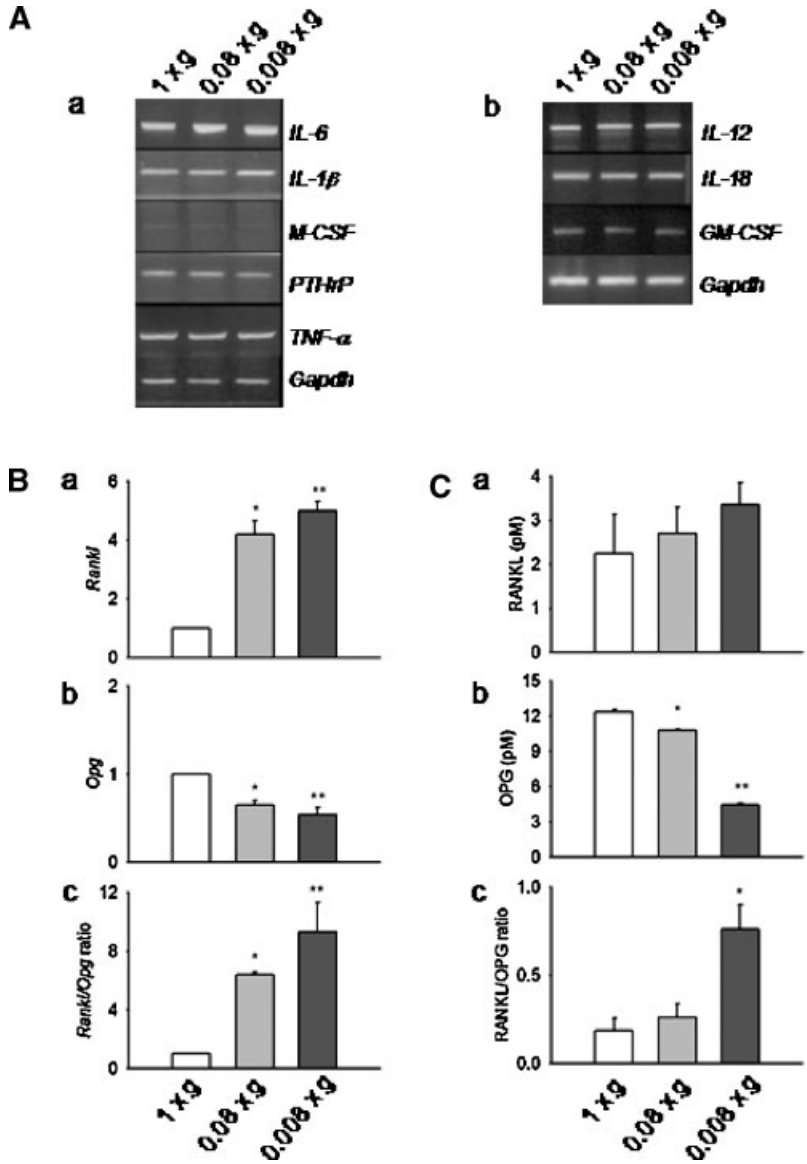

Fig. 2. Osteoblast transcriptional expression of osteoclast-regulating cytokines. **A**, **B**: Osteoblast primary cultures were grown at unit gravity (1*g*) or under 0.08 and 0.008*g* microgravity for 24 h. Cells were then collected and the RNA was extracted and reverse-transcribed. (A) cDNA from osteoblasts of all culture conditions was subjected to PCR for the amplification of the indicated genes (PCR primers and conditions listed in Table I). Results are representative of three evaluations. (B) cDNA from osteoblasts was subjected to comparative real time PCR using the Brilliant® SYBR® Green QPCR master mix and primer pairs and conditions specific for *Rankl*, *Opg* and the house-keeping gene *Gapdh* (Table I). Relative expression of *Rankl* (**a**) and *Opg* (**b**) levels after normalization with *Gapdh*. (a:$^{*}P = 1 \times 10^{-4}$ and $^{**}P = 2 \times 10^{-4}$ vs. 1*g*; b:$^{*}P = 6 \times 10^{-4}$, and $^{**}P = 2 \times 10^{-4}$ vs. 1*g*). **c**: Evaluation of the *Rankl/Opg* ratio. Values are presented as relative expression. Data are the mean ± SD of three independent experiments ($^{*}P = 0.0001$ and $^{**}P = 0.002$ vs. 1*g*). **C**: Conditioned media (CM) from osteoblasts grown for 24 h at 1, 0.08, and 0.008*g* were collected, then soluble RANK-L (a) and OPG (b) were quantified using ELISA kits according to the manufacturer's instructions (b: $^{*}P = 0.005$ and $^{**}P = 0.0002$ vs. 1*g*). (c) Evaluation of the RANKL/OPG ratio ($^{*}P = 0.016$ vs. 1*g*). Data are the mean ± SD of three independent experiments.

We also investigated a potential role of ERK activation on *Rankl* and *Opg* mRNA expression by treatment of osteoblasts under the various gravity conditions in the presence of the PD98059 inhibitor, but observed unremarkable results (not shown).

DISCUSSION

Data collected from long-term space flights demonstrated that astronauts can lose as much bone mass in the proximal femur in 1 month as postmenopausal women on Earth lose in 1 year

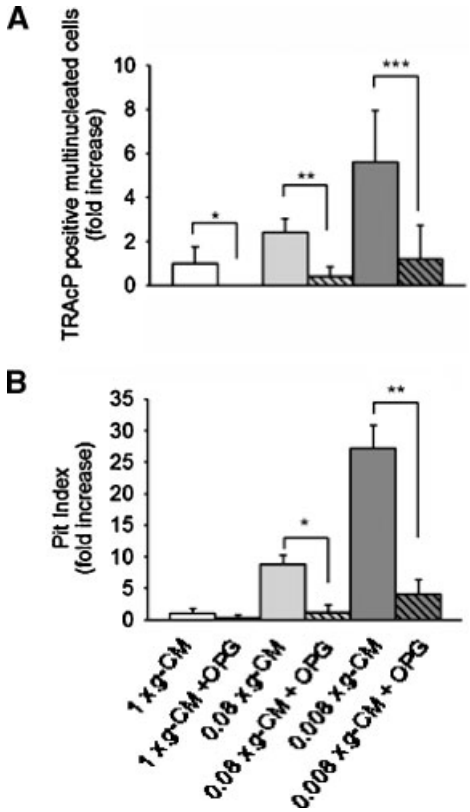

Fig. 3. Effect of OPG on microgravity-induced osteoclastogenesis and bone resorption. **A**: Bone marrow from long bones of 7-day-old mice was cultured onto 24-well multiplates in conditioned media (CM) from osteoblasts grown at 1, 0.08, and 0.008*g* for 24 h. Cultures were treated with OPG (100 ng/ml) or vehicle (5 mM Tris pH 7.5, 0.001% v/v final concentration). After 7 days, cultures were fixed and differentiated osteoclasts were stained for the TRAcP enzyme and counted. Data are expressed as mean ± SD of three independent experiments (*$P = 0.04$; **$P = 0.002$; ***$P = 0.02$). **B**: Bone marrow was cultured as described in (A) onto bone slices. After 7 days, slices were fixed and stained with toluidine blue, and the pit index was calculated. Data are expressed as mean ± SD of three independent experiments (*$P = 0.001$; **$P = 0.007$).

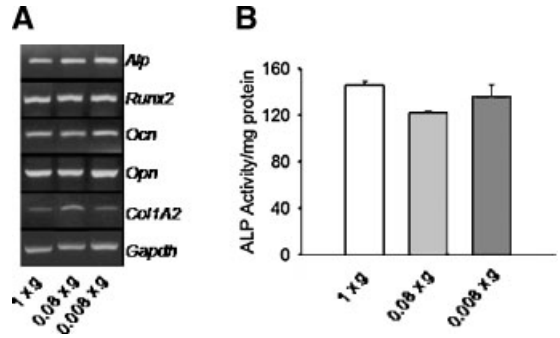

Fig. 4. Effect of microgravity on osteoblast differentiation and matrix protein expression. Osteoblast primary cultures from mouse calvaria were grown at unit gravity (1*g*) or under 0.08 and 0.008*g* microgravity conditions for 24 h. **A**: mRNA was extracted and reverse-transcribed, and the cDNAs were subjected to amplification for the indicated genes following PCR conditions listed in Table I. Results are representative of three evaluations. **B**: 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 ± SD of three independent experiments.

[Cavanagh et al., 2005]. The imbalance between osteoblast bone formation and osteoclast bone resorption during bone remodeling is responsible for bone loss. With regard to the osteoclast function, it has been established a significant increase of bone resorption markers and urinary calcium in humans during space-flights [Smith et al., 2005]. Histomorphometric studies of tibias from rats subjected to space-flight confirmed a reduction of trabecular bone volume, thickness, and number [Durnova et al., 1996]. Moreover, Vico et al. [1987] showed that in unloaded bones the number of osteoclasts/square millimeter of the trabecular surface significantly increased. Similar increase of bone resorption was observed in rats in another ground-based study by Smith et al. [2002].

Starting from these data and based on the well-known effect of osteoblasts on osteoclast differentiation and function we hypothesized that microgravity could affect osteoclastogenesis through regulation of osteoblast activity. Our results provided evidence that under modeled microgravity, osteoblasts released low levels of OPG, with a consequent increase of RANKL/OPG ratio which in turn stimulated mouse bone marrow osteoclast differentiation and activity. Treatment with exogenous OPG reverted the increased osteoclastogenesis observed in the presence of CM harvested from microgravity-cultured osteoblasts. It is interesting to note that while *Rankl* mRNA was several fold increased by microgravity, the release of soluble RANKL in the culture media was modestly incremented, likely reflecting the fact the most of the transcript is translated into membrane-bound RANKL. These results support our hypothesis and settle a rationale for therapeutic trials in space focused on pharmacological intervention that have been successful in preventing and treating osteoporosis in Earth and that could countermeasures for bone loss during long-duration space flight, as it is clear that the osteogenic stimulus from exercise is inadequate to maintain bone mass [Cavanagh et al., 2005]. Among the anti-resorptive

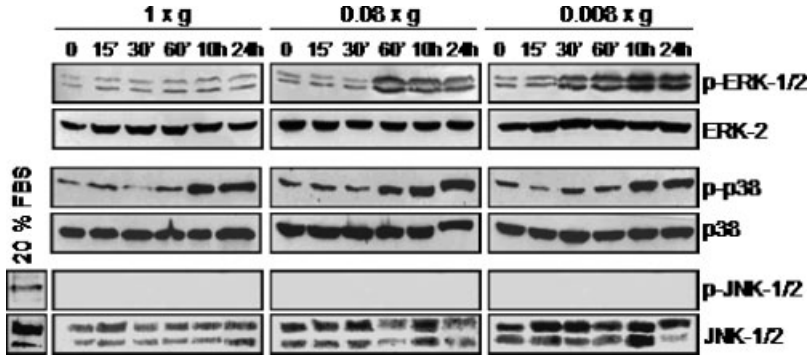

Fig. 5. MAPKs activation. Osteoblast primary cultures were grown at unit gravity (1*g*) or under 0.08 and 0.008*g* microgravity for 15, 30, and 60 min, 10 and 24 h. Total cell lysates were processed by SDS–PAGE and Western blot for the detection of total and phosphorylated fraction of ERK1/2 (**upper panels**), p38 (**middle panels**), and JNK (**lower panels**). An aliquot of cells was treated for 10 min with 20% FBS to show control positive assay for phosphorylated JNK (lower panels). Filters were then stripped and reprobed for the non-phosphorylated proteins as loading control. Similar results were obtained in three independent experiments.

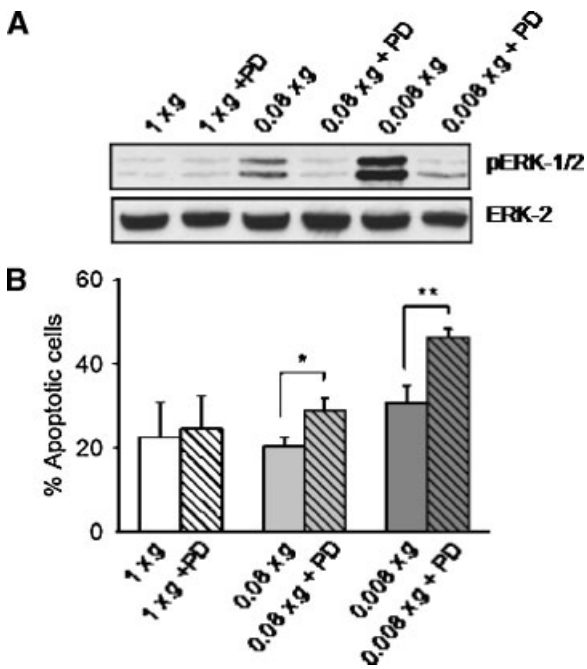

Fig. 6. Effect of ERK1/2 inhibition on apoptosis. Osteoblast primary cultures were grown at unit gravity (1*g*) or under 0.08 and 0.008*g* microgravity for 24 h in the presence of the MEK1/2 inhibitor, PD98059 (50 μM) or vehicle alone (DMSO, 0.01% v/v final concentration). **A**: Cells were lysed in RIPA buffer and 50 μg of proteins were subjected to 12% SDS–PAGE. Filters were immunoblotted with anti-phospho-ERK-1/2 antibody, stripped, and reprobed with anti-ERK-1/2 antibody. Results are representative of three experiments. **B**: 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. 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 are the mean ± SD of three independent experiments (*$P = 0.05$; **$P = 0.03$).

therapies, the RANKL/OPG signaling pathway could thus represent a suitable target for a preventative intervention as it appears that the increase of RANKL/OPG ratio occurs before any detrimental effect on the osteoblast phenotype.

In fact, because microgravity is reported to affect osteoblast function [Bikle et al., 2003] we also evaluated osteoblast differentiation markers such as *Alp* and *Runx2* and the expression genes encoding for the bone matrix protein *osteocalcin*, *osteopontin*, and *collagen 1A2* chain. Surprisingly, we failed to find any effect of microgravity on these parameters. This is in contrast with data from literature that clearly demonstrated a decline in osteoblast gene expression [Carmeliet et al., 1997; Carmeliet et al., 1998; Zayzafoon et al., 2004]. However, these studies have investigated the osteoblast phenotype upon seven or more days of microgravity, whereas in our experiments osteoblasts were maintained under microgravity for 24 h [Carmeliet et al., 1997, 1998; Zayzafoon et al., 2004]. Therefore, our results suggest that the effect of microgravity on osteoclastogenesis via osteoblasts is a rather early event, and that the effect on osteoblast differentiation and function occurs in a later phase. This is consistent with the rapid turnover of osteoclast activity and the slower osteoblast changes generally observed during the process of bone remodeling [Takeda, 2005], and suggests that early pharmacologic intervention with anti-resorptive agents in astronauts during space flights may contribute to reduce bone loss due to early induction of osteoblast-derived osteoclastogenic factors.

Osteoblast activity strongly relies on events regulated by a number of phosphorylating enzymes, including members of the c-Src, PKC, and MAPKs families [Franceschi and Xiao, 2003; Lampasso et al., 2002; Longo et al., 2004]. In our study the expression and activity of these kinases were not affected, except for the MAPK, ERK-1/2. We could not relate ERK-1/2 activation to the increased osteoclastogenesis, because treatment with PD98059, which inhibits the upstream kinase MEK-1/2, failed to influence osteoblast production of *Rankl* and *Opg*. Moreover, the use of PD98059 suggested a role for ERK-1/2 in preventing apoptosis under microgravity. However, we believe that this result is unlikely to completely explain the meaning of ERK-1/2 phosphorylation under microgravity, so it is conceivable that this kinase is involved in further events which at present remain to be elucidated.

In conclusion, we provide in vitro evidence that the effect induced on bone by microgravity could be due, at least in part, to an increased osteoclast differentiation and function via osteoblasts, which happens at a very early stage of microgravity exposition. Although this finding has been obtained ex vivo and in conditions of simulated microgravity, which represent a limitation of the study, it could allow to more deeply understand the molecular mechanisms underlying bone loss in mechanical unloading. This could later be translated into an insight to understand the situation in astronauts as well as in patients with unloading-induced bone loss.

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