Skeletal unloading inhibits the in vitro proliferation and differentiation of rat osteoprogenitor cells

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Kostenuik, Paul J., Bernard P. Halloran, Emily R. Morey-Holton, and Daniel D. Bikle. Skeletal unloading inhibits the in vitro proliferation and differentiation of osteoprogenitor cells. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E1133–E1139, 1997.—Loss of weight bearing in the growing rat decreases bone formation, osteoblast numbers, and bone maturation in unloaded bones. These responses suggest an impairment of osteoblast proliferation and differentiation. To test this assumption, we assessed the effects of skeletal unloading using an in vitro model of osteoprogenitor cell differentiation. Rats were hindlimb elevated for 0 (control), 2, or 5 days, after which their tibial bone marrow stromal cells (BMSCs) were harvested and cultured. Five days of hindlimb elevation led to significant decreases in proliferation, alkaline phosphatase (AP) enzyme activity, and mineralization of BMSC cultures. Differentiation of BMSCs was analyzed by quantitative competitive polymerase chain reaction of cDNA after 10, 15, 20, and 28 days of culture. cDNA pools were analyzed for the expression of c-fos (an index of proliferation), AP (an index of early osteoblast differentiation), and osteocalcin (a marker of late differentiation). BMSCs from 5-day unloaded rats expressed 50% less c-fos, 61% more AP, and 35% less osteocalcin mRNA compared with controls. These data demonstrate that cultured osteoprogenitor cells retain a memory of their in vivo loading history and indicate that skeletal unloading inhibits proliferation and differentiation of osteoprogenitor cells in vitro.

Bone; alkaline phosphatase; c-fos; osteocalcin; mineralization

Microgravity associated with spaceflight leads to a deficit in bone mass in humans and in animals (15, 18). In rats, the osteopenic response to microgravity is associated with decreased osteoblast numbers, decreased bone formation, and delayed bone maturation (4, 15). Similar changes can be induced in the hindlimbs of the rat by hindlimb elevation (3, 4, 6, 7, 27). Although the osteoblast appears to be the principal mediator of osteopenia in these models, the underlying mechanism for osteoblast inhibition is unclear. Skeletal unloading may decrease the number of osteoprogenitor cells or inhibit their proliferation, as suggested by the decrease in osteoblast numbers in unloaded bone (7) and the decreased proliferation of cultured osteoblasts isolated from unloaded bones (12, 27). Osteoblast differentiation may also be inhibited, as suggested by the inhibition of mineralization and maturation of unloaded bone (3, 6) and by the altered expression of genes associated with osteoblast differentiation in unloaded bone (4).

Cultured osteoblasts differentiate through phases of proliferation, organic matrix synthesis, and matrix mineralization, and these phases are accompanied by the sequential expression of various genes. C-fos expression is associated with the proliferative phase of cultured osteoblasts. Alkaline phosphatase (AP) is a marker of early osteoblast differentiation, and is associated with organic bone matrix synthesis before its mineralization. Osteocalcin is expressed by mature osteoblasts in association with matrix mineralization (17). RNA isolated from whole bone demonstrates that skeletal unloading due to spaceflight or hindlimb elevation causes increased AP mRNA expression and decreased expression of osteocalcin mRNA (4). These changes are consistent with the observed decrease in calcium-to- hydroxyproline ratio in bones after spaceflight or hindlimb elevation (6) and suggest a shift toward decreased maturation of bone (3). It is important to elucidate the response of the osteoblast population to skeletal unloading to understand the mechanisms by which bone formation in unloaded bone is inhibited. The effect of skeletal unloading on the expression of c-fos, AP, or osteocalcin mRNA at the osteoblast level has not been previously demonstrated. The present study was conducted to determine whether osteoblasts isolated from unloaded bones would recapitulate in vitro the altered proliferation and differentiation that is suspected to occur in vivo. This question was addressed by employing quantitative competitive polymerase chain reaction (QC-PCR) to measure the expression of c-fos, AP, and osteocalcin mRNA in rat bone marrow stromal cell (BMSC) cultures from unloaded and normally loaded tibiae. Measurements of cell proliferation, AP activity, and mineralization in vitro further demonstrate that the observed changes in gene expression translate into an altered cell phenotype.

METHODS

Animal protocols and tissue processing. Male Sprague-Dawley rats (Bantam-Kingman, Fremont, CA) weighing 125 g were fed standard laboratory rat chow ad libitum (Wayne Lab Blox F-5, James Grain, San Jose, CA) containing 1.4% Ca and 0.97% P and were maintained on a 12:12-h light-dark cycle. A total of 18 rats were divided into three groups such that the average weights were similar between groups. Six control rats (normal weight bearing) were housed in identical cages as the suspended rats. The remaining 12 rats were hindlimb elevated for 2 (n = 6) or 5 days (n = 6). Skeletal unloading was accomplished using the hindlimb elevation model as previously described (6). To effect unloading, rat tails were cleaned with 70% ethanol and tincture of benzoin (American Hospital Supply, San Francisco, CA) was sprayed along the tail and allowed to dry. This protected the skin from
irritation and formed a sticky surface. A 1-cm-wide piece of orthopedic tape (FasTrac, Van Nuys, CA) was attached laterally along each side of the tail to form a loop near the end of the tail. The tail was then wrapped in a mesh netting (Stockinette, American Hospital Supply) followed by strapping tape. The loop of orthopedic tape at the end of the tail was attached to a pulley system allowing the hindquarters of the animal to be lifted off the ground while permitting the animal free movement about its cage through the use of its forelimbs. The initiation of hindlimb elevation was staggered such that all animals were killed on the same day. At the onset of hindlimb elevation, controls (normal weight-bearing animals) were switched from ad libitum feeding to a pair-feeding regimen, wherein the average daily food consumption of the suspended animals was determined by weighing. This amount was provided to the control animals to assure similar weight gain between groups throughout the study. Animals were weighed daily before and during the study. Hindlimb-elevated animals remained elevated during weighing to prevent reloading of the hindlimbs. At the end of the hindlimb elevation period, animals were killed under isofluorane anesthesia by exsanguination from the dorsal aorta, during which the hindlimb-elevated rats experienced no more than 1 min of reloading. Blood was collected in a heparinized tube and immediately assayed for pH and for serum ionized calcium concentration with a calcium-pH analyzer (Ciba-Corning 234 Ca2+/pH Analyzer; Ciba-Corning Diagnostics, Medfield, MA). The right femur from each animal was removed and cleaned of adherent soft tissue and defatted in a Soxhlet apparatus (Fisher Scientific, Santa Clara, CA) using overnight extractions with ether and then with absolute ethanol. The left femur and both tibiae from each animal were removed, cleaned of soft tissue, and used for BMSC culture.

A separate experiment was conducted to examine the effects of hindlimb elevation on BMSC proliferation and AP activity. Rats were hindlimb elevated for 0 or 5 days (n = 12/group), and their tibial marrow cells were removed and combined to produce four independent pools of cells per group, each of which included the left and right tibial marrow cells from three animals. Cells were cultured in six-well plates as described below for up to 28 days to examine the time course of proliferation and AP enzyme activity.

Cell culture methods. To harvest BMSCs, tibiae were briefly immersed in 95% ethanol and then bisected longitudinally with a razor blade. Marrow cells from the left and right tibiae were harvested with a 10-µl pipet and pooled for each animal. Marrow was diluted in 20 ml of α-Eagle’s minimum essential medium with L-glutamine and ribonucleosides (GIBCO, Grand Island, NY) containing streptomycin (100 U/ml), and penicillin-streptomycin (100 U/ml). A single-cell suspension was obtained by repeated passage through a 18-gauge needle, and cell yields were determined with a hemacytometer. Trypan blue exclusion demonstrated that >95% of cells were viable. For tibial cultures, the six sets of cell suspensions from each group were then reduced to three pools of cells for each group, with each pool containing the marrow cells from left and right tibiae from two animals from the same group. Cell populations isolated from the left femur were cultured individually (5 plates/animal, 6 animals/group). Cells were then added to 10-cm tissue culture dishes at 4 × 10^6/dish for tibial cultures and at 8 × 10^6/dish for femoral cultures and were incubated for 24 h at 37°C in a humidified incubator with 5% CO2. A media change at 24 h removed the great majority of nonadherent hematopoietic cells. The media from this point on also contained ascorbic acid (50 µg/ml) and β-glycerophosphate (10 mM, Sigma, St. Louis, MO) to promote and support bone cell differentiation. Media was changed every 2–3 days for the duration of BMSCs culture, which was extended for up to 28 days.

RNA isolation. After 10, 15, 20, and 28 days of culture, total RNA was isolated from each pool of tibial cells with an RNA Stat-60 kit (TelTest, Friendswood, TX). RNA was diluted in diethylpyrocarbonate-treated water, and the concentration and purity was assessed with a UVikon spectrophotometer (Research Instruments International, San Diego, CA). RNA was electrophoresed on a 1% SeaKem agarose gel (FMC Bioproducts, Rockland, ME) containing ethidium bromide and visualized under ultraviolet (UV) light to confirm its integrity. Twenty micrograms of total RNA from each pool were reverse-transcribed into cDNA using oligo(dT) primers and a Gibco Superscript II Reverse Transcription kit (GIBCO).

Cloning of competitor cDNA templates for QC-PCR. Oligonucleotide primers were designed to produce PCR fragments of the rat genes for c-fos, AP, osteocalcin, and glyceraldehyde phosphate dehydrogenase (GAPDH). Primer sets were chosen to flank a unique restriction site within each gene of interest. Each upper strand primer was designed to include a 5′ EcoRI restriction site, and each lower primer contained a 3′ Hind III site. Upper strand primer for c-fos: 5′-TGCATGAATCCGCCAGGCGCTCTGCTCCA-3′; lower strand primer for c-fos: 5′-TGCATAAGCTTCACACTTCCGATTCC-3′; upper strand primer for AP: 5′-TGCATGACCCGCCGGGAGGGCGAGT-3′; lower strand primer for AP: 5′-TGCATAAGCTTAGAGCCCAAAAGGG-3′; upper strand primer for osteocalcin: 5′-TGCCATGAATCGCCGAGCACCATG-3′; lower strand primer for osteocalcin: 5′-TGCCATGAATCGCCGAGCACCATG-3′; upper strand primer for GAPDH: 5′-TGCCATGAATCGCCGAGCACCATG-3′; lower strand primer for GAPDH: 5′-TGCCATGAATCGCCGAGCACCATG-3′.

Oligo-Cloning of competitor cDNA templates for QC-PCR. Oligonucleotide primers were designed to produce PCR fragments of the rat genes for c-fos, AP, osteocalcin, and glyceraldehyde phosphate dehydrogenase (GAPDH). Primer sets were chosen to flank a unique restriction site within each gene of interest. Each upper strand primer was designed to include a 5′ EcoRI restriction site, and each lower primer contained a 3′ Hind III site. Upper strand primer for c-fos: 5′-TGCATGAATCCGCCAGGCGCTCTGCTCCA-3′; lower strand primer for c-fos: 5′-TGCATAAGCTTCACACTTCCGATTCC-3′; upper strand primer for AP: 5′-TGCATGACCCGCCGGGAGGGCGAGT-3′; lower strand primer for AP: 5′-TGCATAAGCTTAGAGCCCAAAAGGG-3′; upper strand primer for osteocalcin: 5′-TGCCATGAATCGCCGAGCACCATG-3′; lower strand primer for osteocalcin: 5′-TGCCATGAATCGCCGAGCACCATG-3′; upper strand primer for GAPDH: 5′-TGCCATGAATCGCCGAGCACCATG-3′; lower strand primer for GAPDH: 5′-TGCCATGAATCGCCGAGCACCATG-3′.

Bone cell cDNA was amplified via PCR using these primer sets to produce the following PCR products: c-fos from nucleotide 230–617 [388 base pair (bp)] product), AP from nucleotide 187–443 (256 bp) product), and GAPDH from nucleotide 171–551 (380 bp) product. These products were gel-purified using a Qiagen II Gel Extraction Kit (Qiagen, Chatsworth, CA) and were then digested with EcoRI and HindIII. All restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Digested PCR products were ligated into EcoRI/HindIII-digested pGEM-4Z plasmid (Promega, Madison, WI) with T4 DNA ligase (New England Biolabs). Ligation products were transfected into competent XL1-Blue cells (Stratagene, La Jolla, CA). Individual bacterial colonies containing each PCR product within the pGEM-4Z vector were identified by EcoRI/HindIII restriction digests, and vector DNAs containing c-fos, AP, osteocalcin, and GAPDH inserts were purified with a Qiaprep Spin Plasmid Miniprep Kit (Qiagen, Chatsworth, CA). These vectors were then linearized with restriction endonucleases, which recognized unique sites within each PCR product insert. C-fos was linearized with Bgl II, AP, and GAPDH were linearized with MscI, and osteocalcin was linearized with BsaI. A 25-bp DNA oligo with compatible ends was then ligated into the linearized vectors. This oligo, which serves as spacer DNA, contains an internal EcoRI recognition sequence and has either Bgl II, MscI, or BsaI sites on both ends.
ends for ligation into the appropriately digested vector. Oligo
for c-fos: 5′-AGATCTAGGATGAGTTTGCATGCGATCTT-3′; oligo for AP and for GAPDH: 5′-TGGCAATTGACTGATATCCTACGATGCTGCCG-3′; oligo for osteocalcin: 5′-CATTCTGCAATGAGGATGCTGAGATT-3′. Ligation products were transfected into XL1-Blue cells as described above. Bacterial
dones were identified that contained these spacer DNA oligos
within the original PCR insert. Identification of these dones
was facilitated by digesting the vector DNA with EcoRI/Hind
III, which cuts the PCR insert out of the vector and also
deaves the PCR insert into two pieces due to the nested EcoR
1 site within the spacer DNA oligo. These competitor vectors
were purified as described above, quantified spectrophotomet-
rally, and diluted to 1 ng/µl.

QC-PCR. QC-PCR is a sensitive and accurate method for
quantifying gene expression and is based on the coamplifica-
tion of an unknown amount of wild-type cDNA with a known
amount of competitor cDNA template (19). A constant amount
of cDNA from each cDNA pool was added to mastermixes
containing PCR Supermix (GIBCO), various known amounts
of individual competitor cDNA templates, and the correspond-
gene-specific primers that were used to clone the competi-
tor cDNA templates. These primers recognize both the com-
petitor RNA template and the corresponding wild-type gene
within the mixed cDNA pool and thus lead to the production
of PCR products that differ in size by 25 bp. The ratio of the
larger competitor PCR product to the smaller wild-type
product reflects the relative concentration of each template in
the PCR reaction. This relationship is linear within at least
one order of magnitude surrounding the point of equivalency
of competitor and wild-type products (unpublished data and
Ref. 19). PCR amplification was performed with an EriComp
Twinblock thermal cycler (EriComp, San Diego, CA), and the
resulting reactions were resolved over a 3.5% NuSieve Aga-
rose gel (FMC Bioproducts) containing 1 µg/ml ethidium bromide.
For each gene, all cDNA pools were analyzed together and resolved on the same gel. Gels were photo-
grahed under UV light and scanned into an Adobe Photo-
shop file (Mountain View, CA) using a UC630 Max Color Scanner (UMAX Data System, Hsinchu, Taiwan). Scanned
images were analyzed densitometrically using NIH Image
1.59 software and the ratios of optical densities of the
competitor and wild-type PCR products were calculated.
These ratios were plotted against the amount of competitor
cDNA template added. Linear regression analysis was used to
calculate the concentration of wild-type product, which repre-
sents the amount of competitor cDNA template that would
give a competitor-to-wild type ratio of 1.00.

The GAPDH gene was used to control for potential varia-
tions in the efficiency of reverse transcription between the
various cDNA pools. GAPDH levels were determined in
tricipate for all cDNA pools, and the data for c-fos, AP, and
osteocalcin gene expression were then normalized to GAPDH
levels and expressed as nanograms per micrograms GAPDH.
Before reverse transcription, RNA aliquots were subjected to
Northern blot analysis with a GAPDH probe (data not
shown). This analysis demonstrated that GAPDH gene expres-
sion was not influenced by hindlimb elevation or by duration
in culture and was thus a valid housekeeping gene to control
for potential variations in reverse transcription efficiency
between the 36 independent cDNA pools.

Cell proliferation and AP activity assays. BMSCs were
isolated from the tibiae of a separate group of rats (n = 12/group) that had been hindlimb elevated for 0 (control) or 5
days. Cells were combined to produce four pools of cells per
group, with each pool including the left and right marrow
populations from three control or hindlimb-elevated rats.

Table 1. Effect of hindlimb elevation on body weight, femur fat-free weight, and serum ionized
calcium concentration

<table>
<thead>
<tr>
<th>Duration of HLE</th>
<th>Fat-Free Femur Weight, mg</th>
<th>Body Weight, g</th>
<th>Serum Ionized Calcium, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Days</td>
<td>273.2 ± 12.7</td>
<td>170.4 ± 2.8</td>
<td>1.403 ± 0.020</td>
</tr>
<tr>
<td>2 Days</td>
<td>269.2 ± 7.7</td>
<td>177.4 ± 8.1</td>
<td>1.483 ± 0.082</td>
</tr>
<tr>
<td>5 Days</td>
<td>246.8 ± 12.0</td>
<td>167.2 ± 4.6</td>
<td>1.486 ± 0.034†</td>
</tr>
</tbody>
</table>

Values are means ± SE. HLE, hindlimb elevation. *Significantly
less than control (0 days HLE) and 2 days HLE, P < 0.05. †Signifi-
cantly greater than control, P < 0.05.

Cells were added to six-well tissue culture plates at 4 × 10³
cells/well under the conditions described above and cultured
for up to 28 days. After various durations of culture, cultures
were fixed for 1 h with 10% Formalin and rinsed with distilled
water. Cell number was determined by staining with crystal
violet as previously described (5). Briefly, fixed cultures were
incubated at room temperature with 0.2% crystal violet in 2%
ethanol for 20 min. The staining solution was then aspirated,
and cultures were rinsed four times with distilled water to
remove unbound stain. Specifically bound nuclear stain
was then eluted with 0.2% Triton X-100, the intensity of which
was measured with a spectrophotometer at 590 nm. The same
destained cultures were then rinsed again with distilled
water and incubated for 15 min at 37°C with 1 ml of a solution
containing equal parts p-nitrophenol phosphate (1× Sigma
104 phosphatase substrate) and alkaline buffer solution
(Sigma 221). The reaction was stopped by adding 0.05 N
NaOH, and the AP activity was measured with a spectropho-
tometer at 410 nm.

In vitro mineralization. After 28 days of culture, 10-cm
dishes containing femoral BMSCs were rinsed with phosphate-
buffered saline and fixed for 1 h with 10% Formalin. After
fixed cultures were rinsed with distilled water, they were
stained for 5 min with 1% alizarin red in 2% ethanol to reveal
mineral. The cultures were then rinsed five times with
distilled water to remove loosely bound stain. The resulting
stained nodules were too numerous to accurately quantify, so
the stain was solubilized for 30 min at room temperature with
0.5 N HCl-5% sodium dodecyl sulfate (SDS). The solubilized
stain was removed from the plates, and the absorbance was
measured in a spectrophotometer at 415 nm. For each
animal, five replicate 10-cm dishes were analyzed and aver-
eged, and the mean values for six animals were averaged for
each group. Preliminary experiments demonstrated that the
absorbance was linearly related to the amount of alizarin red
deluited over the range measured in these experiments.

Statistical analysis. Differences between hindlimb-elev-
ated and normally loaded animals were determined with a
two-way analysis of variance (ANOVA) using SigmaStat
(Jandel Scientific Software, San Rafael, CA).

RESULTS

Five days of hindlimb elevation led to a significant
decline in the fat-free weight of the femur (P < 0.01)
and a small but significant increase in serum ionized
calcium concentration (Table 1). The average body
weight of each group was not different before or after
hindlimb elevation. There was no significant effect of 2
days of hindlimb elevation on these parameters, al-
though serum ionized calcium tended to be elevated.

QC-PCR was used to measure the levels of mRNA
expression of GAPDH, c-fos, AP, and osteocalcin in each

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cDNA pool. The data obtained represents the average of triplicate measures of each gene in each cDNA pool. Each suspension condition consisted of three independent cDNA pools, each of which represented RNA obtained from bone marrow cells isolated from two rats. GAPDH expression, which was used to normalize data as a control for potential differences in the reverse transcription of RNA, was similar among all groups, as assessed by Northern blot analysis and by QC-PCR (data not shown). QC-PCR was then used to determine the GAPDH levels for each cDNA pool, and the expression of c-fos, AP, and osteocalcin was normalized to the GAPDH level.

QC-PCR analysis demonstrated that the expression of c-fos mRNA was significantly decreased in osteoprogenitor cells isolated from animals that were hindlimb elevated for 5 days (Fig. 1). Decreased c-fos mRNA expression was not apparent at day 10 in cultures from unloaded rats, but, at days 15 and 20, the cells from the 5-day hindlimb-elevated group had 50% lower c-fos levels. At day 28, c-fos mRNA levels in these cultures were reduced by 80% vs. controls. Two days of hindlimb elevation caused a consistent decrease in c-fos mRNA expression compared with controls, but the overall 42% decrease throughout the culture period did not reach statistical significance (P = 0.09).

AP mRNA expression was significantly elevated in osteoprogenitor cells isolated from 5-day hindlimb-elevated rats compared with controls (Fig. 2). Most of this increased expression was manifest during the early time points in culture (10 and 15 days), and the overall increase throughout the culture period was 61% compared with controls (P < 0.05). Two days of hindlimb elevation led to an insignificant 26% increase in AP mRNA expression compared with controls. Osteocalcin mRNA expression was decreased overall by 35% in cultures from the 5-day hindlimb group, whereas the 2-day hindlimb-elevated group showed nonsignificant reductions in osteocalcin mRNA compared with controls (Fig. 3). Because osteocalcin mRNA expression is associated with mineralization, we used an extra set of cultures in 10-cm dishes to examine in vitro mineralization. Alizarin red staining binds precipitated calcium salts and was used to reveal mineral in 28-day-old cultures. The mean absorbance of solubilized stain in the 5-day unloaded group was 40% lower than in the control group (P < 0.05), whereas the 2-day unloaded group was not significantly lower than controls (20%) (Fig. 4).

Five days of hindlimb elevation were sufficient to cause significant changes in the differentiation and mineralization of cultured osteoprogenitor cells. We therefore examined the effects of 5 days of hindlimb elevation on the proliferation and AP activity of these cells. Cell number was determined by crystal violet staining, and the initial plating density of the different groups (at days 3, 4, and 5 of culture) was shown to be the same in the two groups (see Fig. 5, inset). By day 7 there was a small but consistent decrease in cell number in the cultures from hindlimb-elevated rats compared with controls, and this difference tended to...
get larger over time. The cultures from hindlimb-elevated rats reached quiescence by day 21, as evidenced by a plateau of the growth curve, whereas, in the control cultures, cell number was still increasing at day 28. The decreased cell number in cultures from hindlimb-elevated rats was significant (P < 0.05, 2-way ANOVA). These same cultures were then assayed for AP activity in a novel adaptation of the standard Sigma colorimetric enzyme activity assay. The standard assay involves solubilizing the cells with detergent, measuring enzyme activity, and expressing activity as a function of total protein or another variable. The cell membrane-associated enzyme activity was found to be stable in fixed cultures before and after crystal violet staining and destaining, so fixed cultures were incubated with AP substrate without solubilizing the cells. AP activity was then expressed by dividing the absorbance of liberated phenol (410 nm) by the previously determined absorbance of crystal violet (590 nm). Hindlimb elevation led to a significant decrease in AP activity at all time points compared with control cultures (Fig. 6A). When AP activity is expressed on a per cell basis, the cultures from hindlimb-elevated rats are still significantly lower than controls (Fig. 6B).

**DISCUSSION**

Gravity induces mechanical loading in weight-bearing bones, which is necessary for the long-term maintenance of normal skeletal architecture. The nature of the biological signal that mediates mechanotransduction in bone is poorly understood, but it is clear that skeletal unloading significantly alters bone metabolism. Skeletal unloading decreases osteoblast number, bone formation rate, bone mass, bone maturation, and mechanical strength (3, 6, 7, 15, 22, 25, 26). Cells of the osteoblast lineage are responsible for bone formation and are known to respond to mechanical loading (8, 14). These cells, which include osteoprogenitor cells, osteoblasts, and osteocytes, are the most conspicuous candidates for mediating the skeletal response to unloading.
It is important to elucidate the biological response of these cells to skeletal unloading before one can understand the nature of the mechanical signals to which they respond.

We have hypothesized that skeletal unloading causes decreases in osteoblast proliferation and differentiation, which leads to a decrease in osteoblast numbers and a shift within the osteoblast population to cells that are less capable of producing mature, well-mineralized bone matrix. Several studies have suggested that skeletal unloading decreases osteoblast proliferation. The decreased number of osteoblasts observed in unloaded bone (7) may indicate a decrease in the proliferation or recruitment of osteoblast precursors from bone marrow or from the peristeme. In fact, BMSCs isolated from unloaded bone have been shown to proliferate more slowly than BMSCs from normally loaded bone (Refs. 12 and 27 and present study). These results could reflect deficits in the absolute number or recruitment of osteoblast precursors or decreased proliferation of committed osteoprogenitor cells. A previous study reported that skeletal unloading led to a significant decrease in osteoprogenitor cell number as early as day 6 of culture, but that thymidine incorporation was not different from controls at this time point (9). The present data suggest that the number of potential osteoprogenitor cells is different in unloaded bone, because the number of adherent stromal cells measured in culture immediately after isolation from unloaded bone is virtually identical to control bone. The sensitivity of the crystal violet assay permits the quantification of as few as 500 cells (5), and as such we are able to accurately measure cell number before they begin to proliferate. This is demonstrated by a lack of change in cell number from day 3 to day 5 in culture. Proliferation is evident by day 7, which coincides with the onset of decreased cell number in cultures of hindlimb-elevated rats. In addition to a decrease in proliferation rate, the total proliferative potential of cells from unloaded bone may also be reduced, as evidenced by an earlier plateau of the growth curve (at 21 days in cultures from hindlimb-elevated rats, and >28 days in control cultures).

The decreased proliferation of osteoprogenitor cells isolated from unloaded bone is consistent with the observed decrease in the expression of c-fos, a gene that is associated with osteoblast proliferation (11, 17). The unloading-induced decrease in c-fos expression suggests that the decreased proliferative activity of BMSCs occurs throughout their differentiation pathway and is not restricted to the earliest stages of osteoprogenitor recruitment. The 80% decrease in c-fos expression at day 28 in cultures from 5-day hindlimb-elevated rats is further evidence that these cells may have reduced proliferative potential and may reach quiescence earlier than controls.

Immature osteoblasts synthesize organic bone matrix, which is then mineralized as differentiation progresses. Immature osteoblasts are characterized by their high levels of type I collagen production and high AP gene and protein expression (1, 13, 17, 24). Inhibition of osteoblast differentiation may be manifest by a relative increase in the population of these immature cells. Weightlessness has been shown to inhibit the differentiation of osteoblasts in vivo (20, 21), and this inhibition may explain changes that occur at the whole bone level after skeletal unloading. These changes include increased collagen concentration (16), decreased mineralization (6, 15, 25), decreased calcium-to-hydroxyproline ratio (6, 23), and increased AP mRNA in unloaded bone (4). In the present study, we have demonstrated a specific increase in level of AP mRNA expression in cultured BMSCs isolated from tibiae that were unloaded for 5 days. This result is consistent with the increased level of AP mRNA observed in whole rat tibiae unloaded by spaceflight and by hindlimb elevation (4).

AP enzyme activity was reduced by hindlimb elevation, both at the level of the whole culture and on a per-cell basis. AP activity has been previously reported to be reduced in cells isolated from femurs that were unloaded by sciatic neurectomy (9). In the present study, the apparent discrepancy between AP mRNA expression and AP enzyme activity has several potential explanations. The increased AP mRNA induced by unloading may not be quantitatively translated into protein, or the catalytic activity of the AP protein may be inhibited after unloading. Furthermore, AP mRNA levels have been previously shown to be influenced by an inducible mRNA-stabilizing factor (10), indicating a complex system of posttranscriptional regulation of AP. It is tempting to speculate that the regulation of AP enzyme activity may contribute to the osteoblast response to unloading.

The ability to form mineralized matrix in culture is perhaps the most important index of well-differentiated osteoblasts. The increased expression of AP mRNA and decreased expression of osteocalcin mRNA in unloaded bones suggests that a greater proportion of osteoblasts remain in the early stage of matrix synthesis and are delayed in their normal progression to active mineralization. We addressed this question by staining 28-day femoral BMSC cultures with alizarin red, which reveals mineralized bone cell nodules. BMSCs isolated from 5-day unloaded femurs formed 40% less mineral compared with controls, which indicates that skeletal unloading in vivo leads to impaired mineralization in vitro. This result is consistent with in vivo data demonstrating that skeletal unloading causes a decreased mineralization and an increased calcium-to-hydroxyproline ratio (6, 23, 25). These data support the hypothesis that skeletal unloading decreases osteoblast differentiation and demonstrate the utility of this model for studying the effects of weightlessness on osteoblast differentiation.

These data contrast with those obtained by Machwate et al. (12), who reported that 14 days of hindlimb elevation did not alter the osteoblast phenotype in vitro. In our study, animals were hindlimb elevated for 2 or 5 days. Previous studies indicate that the effects of skeletal unloading on bone formation in the growing rat are transient. After 5 days of hindlimb elevation, bone formation, calcium accumulation in bone, and
serum 1,25(OH)₂D₃ concentration are decreased. However, these parameters all return toward normal levels after 12–14 days of continuous hindlimb elevation, indicating a recovery of bone formation despite continued unloading in these young animals (6, 7). Therefore, osteoblasts isolated from tibiae after 14 days of hindlimb elevation might not be expected to differ substantially from control cells.

In conclusion, we have demonstrated that the osteoprogenitor cells isolated from the tibiae of rats that were hindlimb elevated for 5 days expressed significantly less c-fos mRNA, more AP mRNA, and less osteocalcin mRNA than did cells from normally loaded tibiae. Changes in gene expression with 5 days of unloading were also accompanied by decreases in proliferation, AP activity, and mineralization compared with control cultures. The changes in osteoblast gene expression and phenotype after unloading are consistent with a shift toward a less mature osteoblast population, suggesting an inhibition of osteoprogenitor cell differentiation with skeletal unloading. These data also demonstrate that cultured osteoprogenitor cells retain a “memory” of their prior in vivo loading history, indicating that this model is valuable for studying the effects of skeletal unloading on osteoblast function in vitro.

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