Effect of hypophysectomy on the proliferation and differentiation of rat bone marrow stromal cells

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Yeh, James K., Jodi F. Evans, Meng-Meng Chen, and John F. Aloia. Effect of hypophysectomy on the proliferation and differentiation of rat bone marrow stromal cells. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E34–E42, 1999.—Conditions such as estrogen deficiency, skeletal unloading, and aging have all been demonstrated to have various effects on the proliferation and differentiation of bone marrow stroma-derived osteoprogenitor cells. Here we have sought to examine the effects of pituitary hormone deficiency on the proliferation and differentiation of these osteoprogenitor cells using the hypophysectomized (HX) rat as a model. In the present study, we use an in vitro culture system to examine the effects of HX on the osteogenic potential of rat bone marrow stroma. With the intact animal as a control, we used [3H]thymidine incorporation and cell number as indexes of proliferation. We also measured alkaline phosphatase enzyme activity, relative levels of osteocalcin expression with RT-PCR, and osteopontin and bone sialoprotein steady-state levels by Northern blot to delineate the effect on differentiation. Our results indicate that osteoprogenitor cells exposed to a pituitary hormone-deficient environment in vivo demonstrate an enhanced proliferative capacity and also exhibit an augmented expression of differentiation markers when exposed to an optimal environment in vitro.

pituitary hormone; osteoblasts; osteogenesis; alkaline phosphatase; calcium

THE SKELETON OF VERTEBRATES is capable of extensive regeneration (modeling and remodeling), which serves to maintain its health and strength. To retain this capacity, it must possess precursor cell populations capable of differentiating into fully functional osteoblasts and osteoclasts, and the quantity of cells in these precursor pools must be maintained through proliferation (5). Results of studies performed on cells from animals are consistent with the hypothesis that the renewal of the osteoblast population at the bone surface is dependent on the recruitment, proliferation, and differentiation of osteoprogenitor cells (18, 31). It has been determined that cells of the osteogenic lineage, which includes both osteoblasts and chondroblasts, are derived from multipotential mesenchymal stem cells present in the bone marrow stroma (2, 15, 19).

In vitro culture systems have been established in an effort to characterize the stromal system of bone. Conditions permissive for osteoblast differentiation from marrow stromal cells have been tested extensively. These cells, when grown in the presence of β-glycerophosphate, ascorbic acid, and dexamethasone, have been proven to form mineralized bone nodules (4, 6, 22, 28). Three stages of development have been delineated for these primary derived osteoblasts: proliferation, extracellular matrix (ECM) maturation, and ECM mineralization (2, 26, 28). As the osteoprogenitor develops into the preosteoblast and osteoblast under these culture conditions, it expresses various bone-specific markers in a temporal manner. For example, type I collagen and alkaline phosphatase (AP) expression is relatively high at early stages but then decreases as mineralization occurs; osteopontin (OPN) is the first matrix protein to appear followed by bone sialoprotein (BSP) and osteocalcin (OCN), with BSP detected first and OCN appearing with mineralization (2).

Conditions such as estrogen deficiency, skeletal unloading, and aging have all been demonstrated to have various effects on the proliferation and differentiation of osteoprogenitor cells derived from bone marrow stroma. Here we have sought to examine the effects of pituitary hormone deficiency on the proliferation and differentiation of bone marrow-derived osteoprogenitor cells with the hypophysectomized (HX) rat as a model. The HX rat has been used as a model for the effects of pituitary hormone deficiency on bone for many years. Systemically, it results in a suppression of weight gain, a reduction in lean mass, and decreased skeletal growth (8, 14, 30, 33). One of the most striking effects of HX demonstrated in vivo is a cessation of longitudinal growth (14, 30, 33). Hyperphosphase has also been shown to result in cancellous bone loss because of the suppression of longitudinal growth-dependent bone gain and the inhibition of tissue-based bone turnover, with more suppression occurring in bone formation than in resorption (8, 33). In cortical bone, HX results in suppressed radial growth-dependent bone gain, which is associated with a decrease in modeling-dependent bone formation (8). Studies focusing on de novo bone differentiation have yielded similar results. Not only does HX markedly reduce and delay osteogenesis, but also the quality of new bone formed is deficient in the absence of hypophysial hormones (21, 29). On examination of the bone surface of the HX animal, we have discovered a reduction in the osteoblast and osteoclast population compared with an intact age-matched control.

Currently, it is not known whether the reduction in osteogenesis at the bone surface can be attributed to a reduction in the capacity of osteoprogenitors to proliferate and develop into mature osteoblasts. In the present study, we use an in vitro culture system to examine the effects of HX on the osteoprogenitor potential of rat bone marrow stroma. With the intact animal as a...
control, we used [3H]thymidine incorporation and cell number as indexes of proliferation. We also measured AP enzyme activity, relative levels of OCN expression with RT-PCR, and OPN and BSP steady-state levels by Northern blot to delineate the effect on differentiation. Our results indicate that osteoprogenitor cells exposed to a pituitary hormone-deficient environment in vivo demonstrate an enhanced proliferative and differentiative capacity in vitro when their environment is again optimal.

MATERIALS AND METHODS

Animal care. Age-matched HX and intact control female, 8-wk-old Sprague-Dawley rats were purchased from Hilltop where the hypophysectomy surgeries were performed. On arrival, 3 days postoperatively, and throughout the experiment, the HX rats were given 3% sucrose water and allowed free access to a standard pelleted chow diet (Rodent Laboratory Chow 5001, Ralston Purina, St. Louis, MO). Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and animal protocols were approved by the Laboratory Animal Care Committee of Winthrop University Hospital.

Histomorphometric analysis. Rats were labeled with 8 mg/kg of calcein subcutaneously (Sigma, St. Louis, MO) at 3 days before being killed. The left and right tibiae were removed and cut with an Isomet saw (Buehler, Lake Bluff, IL). The proximal section of each right tibia was stained by Villanueva osteochrome bone stain (Polysciences, Warrenton, PA) for 5 days and then processed for methyl methacrylate embedding without decalcification (33). The specimens were destained and dehydrated with sequential changes (70, 95, and 100%) of ethanol solution and xylene and then embedded in methyl methacrylate (Eastman Organic Chemicals, Rochester, NY). Proximal tibiae were frontal-cut longitudinally with a diamond wire histo-saw (Delaware Diamond Knives, Wilmington, DE) at a thickness of 40 µm and then coverslipped with Eukitt (Calibrated Instruments, Hawthorne, NY).

Histomorphometric parameters of cancellous bone of the proximal tibia were measured with a digitizing morphometry system (Osteometrics, Atlanta, GA) and the nomenclature standard (20) as described previously (33). Briefly, the measured parameters included total tissue area, trabecular bone area and perimeters, and calcein labeled perimeters in the secondary spongiosa of the metaphysis in the region 1–4 mm proximal to the growth plate-metaphysial junction. These parameters were then used to calculate cancellous bone volume, total volume, and labeled surface tissue area. The left tibia was removed and decalcified for 5 wk at 4°C in 10% EDTA containing 7.0% sucrose (pH 7.2). After a thorough wash in PBS solution, the decalcified bones were dehydrated in graded alcohol and embedded in 2-hydroxyethyl methacrylate (JB4-Plus embedding kit, Polysciences). Polymerization was carried out at 4°C in a cold room overnight. Polymerized blocks were kept in a desiccator at 4°C until sectioning. Sections of 4-µm thickness were cut with a microtome (2040 Reichert-Jung, Cambridge Instruments, Buffalo, NY) and then mounted on glass slides. Tartrate-resistant acid phosphatase was localized with a commercially available kit with α-naphthyl phosphate disodium salt as substrate (Sigma) (33).

Cell culture. Both femoral and tibial bones were removed from the rats at 6 wk post-HX. Age-matched intact rats served as the control group. Under aseptic conditions, the bones were dissected free of soft tissue, and the epiphysial cartilage was scraped away with a scalpel and discarded. The distal end of the bones was then cut open, and bone marrow was flushed out with 10 ml α-MEM medium (without phenol red) supplemented with 10% FBS, antibiotic-antimycotic, 50 µg/ml ascorbic acid, and 2 mM L-glutamine (basal medium) with an 18-gauge needle for the femur and a 22-gauge needle for the tibia. The bone marrow cells were pooled from each of the four bones of each animal, and a single cell suspension was created by repeated flushing through an 18-gauge needle followed by a 22-gauge needle. For the initial proliferation experiments, cells were seeded at a density of 3.5 × 10^5/cm² and allowed to attach for 3 days before the removal of nonadherent cells. Differentiation experiments were carried out in the above medium supplemented with 10 mM β-glycerophosphate and 10^−8 M dexamethasone (differentiation medium), and initial plating was at the same density as in the proliferation experiments. Cell numbers were determined with a hemocytometer.

Adherent and nonadherent cell fractions. Bone marrow cells were plated at a density of 3.5 × 10^5/cm². After a 1-day initial attachment period, the nonadherent cells in the medium were removed and cultured in separate dishes, and then the medium was replaced with either fresh basal or differentiation medium. Cell number was determined after trypsinization with a hemocytometer.

[3H]Thymidine incorporation. Bone marrow cells were plated as stated above and cultured in basal medium. The medium was replaced after 72 h, and the adherent cells were cultured for another 4 days, and the medium was changed every 2 days. One microcurie of [3H]thymidine (37 Ci/mmol, Amersham, Arlington Heights, IL) was added to each well, and the cells were cultured for another 4 h. The medium was then removed, and the cells were washed with PBS and detached with 0.05% trypsin in PBS. The cells were collected by centrifugation, lysed with 0.1 N sodium hydroxide, and [3H]thymidine incorporation was measured with a scintillation counter and expressed in counts per minute.

AP activity. On day 14 of culture, the cells were washed with 1× PBS and fixed with ice-cold 95% ethanol (EtOH). The cells were then washed with distilled H₂O and allowed to equilibrate in 20 mM bicarbonate buffer, pH 8.8, followed by a 20-min incubation at room temperature with 1 mg/ml p-nitrophenylphosphate in bicarbonate buffer with continuous agitation. p-Nitrophenol in the supernatant was then measured spectrophotometrically at 405 nm, and the results were expressed as nanomoles per minute per 10^5 cells. Histochimical staining for AP was performed with the Sigma 85L-1 kit according to the directions of the manufacturer. Cell number for the AP cultures was obtained by the method of Currie (9). After the AP activity of the cells was measured, the cells were fixed again in 10% Formalin for further mechanical stability. They were then washed once with tap water and twice with borate buffer (10 mM, pH 8.8), stained with 1% methylene blue in borate buffer for 15 min, and then rewarmed several times with tap water followed by borate buffer. Methylene blue-positive colony-forming units (CFU) were used as a measure of total CFU-fibroblast (f) colony numbers. Bound methylene blue was then eluted with 1% HCl, and the
absorbance was read at 650 nm. Cell number was then determined by comparison with a standard curve.

Calcium. At day 21 of culture, calcium in the cell layers was extracted with 1% HCl, and calcium content was measured with atomic absorbance. Mineralized nodules were histochromically stained at day 18 of culture with alizarin red, pH 6.2, and nodules were counted manually (24).

RT-PCR for semiquantitation of OCN expression. Bone marrow cells were initially plated at a density of $5 \times 10^5$/cm$^2$ in 60-mm dishes and allowed to adhere to the dish for 5 days before the nonadherent cells were removed and the basal medium was replaced with differentiation medium. The cells were then extracted for RNA at 7, 9, 12, 14, and 16 days of culture with Trizol Reagent (Life Technologies) following the recommendations of the manufacturer. RNA (1 µg) was used in each reaction and was first treated with DNase I (Life Technologies). The RNA was then reverse transcribed under the following conditions: 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 20 mM DTT, 3 mM MgCl$_2$, 0.5 mM each dGTP, dATP, dTPP, and dCTP, 1.25 U RNase inhibitor (Life Technologies), and 50 U M-MLV reverse transcriptase (Life Technologies). The reaction was incubated at 37°C for 2 h followed by 100°C for 10 min and then immediately placed on ice. Aliquots (10 µl) of the reverse transcription reaction were used in the simultaneous PCR reactions for rat OCN and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reactions were carried out under the following conditions: 20 mM Tris·HCl (pH 8.0), 50 mM KCl, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 2.5 mM MgCl$_2$, 1.25 U Taq polymerase (Life Technologies), and 50 pmol each of forward and reverse primers. Primer sequences were as published in Fleet and Hock (12). Reactions were carried out with a Gene-Amp PCR system 2400 thermocycler (Perkin-Elmer, Norwalk, CT). Cycling parameters were as follows: 95°C for 5 min, followed by 20 cycles of 95°C for 15 s, 55°C for 1 min 28 s, 72°C for 45 s, with a final 72°C hold for 7 min. Twenty cycles of PCR were determined to be within the linear range for both OCN and GAPDH.

Quantitation of PCR products and confirmation of sequence identity were performed by Southern blot analysis followed by chemiluminescent detection with a modification of the method of Fleet and Hock (12). Briefly, the Genius nonradioactive detection kit (Boehringer Mannheim, Indianapolis, IN) was utilized with slight modification. The PCR reactions were separated on 2.5% agarose gels. After electrophoresis, the gels were denatured, neutralized, and transferred to Biodyne B membrane (Life Technologies) with 10× standard sodium citrate (SSC) overnight. DNA was fixed to the membrane by baking at 80°C for 1 h. Membranes were prehybridized in 50% formamide, 5× SSC, 5% blocking reagent (Boehringer Mannheim), 0.1% N-laurylsarcosine, and 0.02% SDS for at least 4 h at room temperature. Approximately 15 pmol of 3′-end digoxigenin-linked ddUTP-labeled probe (nonradioactive 3′-end labeling kit from Boehringer Mannheim) per 100 cm$^2$ membrane were added to the prehybridization buffer and allowed to hybridize overnight. OCN was hybridized at 31.8°C and GAPDH at 23°C as described in Fleet and Hock (12). The sequences of the nested oligonucleotide probes were 1) rat OCN, 5′-GTCATTAC-CACCATCTACTCGCCCTC-3′ and 2) rat GAPDH, 5′-CTAAG-CAGTTGTTGTTGACAGA-3′ (Midland Certified Reagent). After hybridization, the membranes were washed twice at room temperature in 2× SSC-0.1% SDS for 30 min followed by one 15-min wash in 0.1× SSC-0.1% SDS. Detection of the digoxigenin probe was carried out at room temperature. Membranes were washed in buffer 1 (0.15 M NaCl and 0.1 M maleic acid, pH 7.5) for 5 min followed by a 1-h incubation in buffer 2 (buffer 1 with 1% blocking reagent). A 1:5,000 dilution of the anti-digoxigenin-AP conjugate was then made in buffer 2, and the membrane was incubated in this solution for 30 min followed by two washes, 15 min each, in buffer 1 and a 5-min wash in buffer 3 (0.1 M Tris, 0.15 M NaCl, and 50 mM MgCl$_2$, pH 9.5). A final incubation in a 1:100 dilution of CDP-Star in buffer 3 for 5 min was performed, and the membranes were exposed to Fuji NIF film for 25 s to 5 min. Band intensities were measured with the SigmaGel computer program (Jandel Scientific Software, San Rafael, CA) and an HP Desk Scan II scanner. OCN expression was normalized to GAPDH.

Northern analysis. Total cellular RNA was extracted from cultures as stated above, and 15-µg aliquots were fractionated on 1.2% agarose-formaldehyde gels. The RNA was then transferred to Biodyne B membrane (Life Technologies) with 20× SSC overnight. Membranes were prehybridized in Hybriol I (Oncor, Gaithersburg, MD) and 0.5 mg/ml sonicated salmon sperm DNA at 42°C for 2 h. The following cDNA probes were used: a 1.0-kb mouse OPN Ecor I fragment, a 1.0-kb mouse BSP Ecor I fragment (generous gifts of Dr. David Rowe, University of Connecticut, Farmington, CT), and a 1.2-kb Ecor I human GAPDH fragment (Clonetech, Palo Alto, CA). Probes were labeled with 32P-dCTP (NEN, Boston, MA) with the RadPrime DNA labeling system (Life Technologies). Membranes were hybridized overnight at 42°C followed by two room temperature washes in 2× SSC-0.1% SDS for 20 min each, one room temperature wash with 0.2× SSC-0.1% SDS for 15 min, and a final wash with 0.2× SSC-0.1% SDS for 15 min. The membranes then exposed to Fuji NIF film overnight with intensifying screens at −80°C. Between hybridizations,bound probes were removed with 0.1% SDS at 80°C. Band intensities were measured with the SigmaGel computer program (Jandel Scientific Software) and an HP Desk Scan II scanner. Expression levels were normalized to GAPDH expression.

RESULTS

Body weight and histomorphometric data. At 6-wk postsurgery, as previously demonstrated, the body weight of the HX rats remained within a 5% range of their initial presurgery weight, whereas the age-matched intact control rats experienced a 32% gain in weight (Table 1). The cancellous bone volume of the proximal tibia was significantly decreased by 6-wk post-HX compared with the intact control group. Using calcein labeling to examine the osteoblast population on the bone surface, we found that the osteoblast number was decreased by HX. Furthermore, the osteo-

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Animal Wt. g</th>
<th>Total Bone Volume (BV/TV) %</th>
<th>Osteoclasts, cells/mm$^2$</th>
<th>Osteoblast Surface, mm/mm$^2$</th>
</tr>
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<tbody>
<tr>
<td>HX</td>
<td>268 ± 21.4</td>
<td>22.6 ± 4.0</td>
<td>7.38 ± 1.0</td>
<td>1.69 ± 0.46</td>
</tr>
<tr>
<td>Intact control</td>
<td>193 ± 13.5</td>
<td>5.9 ± 2.8</td>
<td>3.26 ± 0.6</td>
<td>0.46 ± 0.29</td>
</tr>
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Values are means ± SD of 10 rats/group. Total bone volume values are tibial cancellous bone volume (BV) as a percentage of total tibial volume (TV). *Significant difference between age-matched intact control and hypophysectomized (HX) rats (P < 0.05) as determined by Student's t-test.
clast number per millimeter of growth plate was also decreased to less than one-half of that of the intact animals.

Characterization of bone marrow stromal cell proliferation under basal and differentiating conditions. Total marrow stromal cells recovered from the HX animals were significantly less than total numbers recovered from the intact animals (3.35 \( \pm \) 0.57 \( \times \) 10^6 cells HX vs. 4.57 \( \pm \) 0.77 \( \times \) 10^6 cells control). These data represent the total number of cells recovered from two femora and two tibiae (n = 10). However, initial plating densities were the same in both groups throughout the experiment. After 7 days of incubation in basal medium, the 24-h \(^{3}H\)thymidine incorporation was 88% greater in the HX group, but the cell number per dish did not differ from the intact control (Table 2). However, by 14 days of culture in basal medium, the cell number and total CFU-f were both higher (81 and 70%, respectively) in the HX group compared with the intact control. When the initial 3-day attachment period was followed by the addition of differentiation medium, cell number was still greater in the HX group. By day 8 under differentiating conditions, it was already 67% more than the intact control group, and by day 14 it was 90% greater (Table 3). CFU-AP and CFU-f colony numbers, after 14 days of incubation in differentiation medium, were also greater in the HX vs. intact control

Table 3. Proliferation characteristics of bone marrow stromal cells from intact control and HX rats under differentiating culture conditions

<table>
<thead>
<tr>
<th>Assay</th>
<th>Intact Control</th>
<th>HX</th>
</tr>
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<tbody>
<tr>
<td>No. of cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8 (( \times ) 10^6)</td>
<td>2.1 ( \pm ) 0.13</td>
<td>3.5 ( \pm ) 0.23</td>
</tr>
<tr>
<td>Day 14 (( \times ) 10^6)</td>
<td>8.6 ( \pm ) 0.24</td>
<td>16.3 ( \pm ) 0.85*</td>
</tr>
<tr>
<td>CFU-AP colony, day 14</td>
<td>21.3 ( \pm ) 6.45</td>
<td>33.7 ( \pm ) 6.3*</td>
</tr>
<tr>
<td>CFU-f colony, day 14</td>
<td>34.7 ( \pm ) 10.9</td>
<td>41.3 ( \pm ) 7.88*</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SD of 6 replicate cultures from 4 different animals/group. CFU-alkaline phosphatase (AP) and CFU-f colony values were in numbers of colonies. Cells were allowed to attach to culture dishes for 3 days before nonadherent cells were removed and basal medium was exchanged for differentiation medium. *Significant difference between age-matched intact control and HX groups as determined by Student’s t-test.
Characterization of bone marrow stroma-derived osteoprogenitor cell differentiation. **Cellular indexes.** When AP activity was measured at day 14 of culture under differentiating conditions, the HX cultures demonstrated a greater activity (at least +110%) in all cell fractions (Fig. 2). Mineralized nodules were counted at day 18, and calcium content was measured at day 21 of incubation. These differentiation parameters were also greater in the HX cultures vs. the intact control cultures in all fractions analyzed. The most significant difference was found with the 1-day adherent fraction, where the intact control cultures had developed <10% the number of nodules and only 15% of the level of calcium as the HX group (Fig. 3). Again these results indicate that both the committed preosteoblasts and the noncommitted stromal cells in the HX cultures have an enhanced sensitivity to differentiating conditions.

**Molecular indexes.** Once we found that the cellular markers of osteoblast differentiation were enhanced in the HX bone marrow stromal cell cultures, we sought to examine a few osteogenic markers to determine whether they too would be enhanced. RT-PCR was used to compare relative levels of OCN mRNA levels. Initially, we analyzed OCN expression levels in 1-day adherent and 5-day adherent cultures after 12 days of incubation. The 12-day time point was used because it has been demonstrated to be near the beginning of matrix maturation and the point at which OCN expression can first be detected in stromal cell-derived osteoblast cultures (16, 32). In both the 1-day and 5-day adherent cultures, the HX group demonstrated higher relative OCN expression at day 12 (3× and 1.7×, respectively) (Fig. 4A). The nonadherent fractions were not analyzed for OCN expression. The significant difference in OCN expression between the control and HX cultures prompted us to investigate temporal differences in expression. After a 5-day initial seed, total RNA was extracted from HX and intact control cultures at days 7, 9, 12, 14, and 16 and analyzed for relative OCN expression (Fig. 4B). No significant difference was seen until day 12, and by day 16, the difference had increased to the point at which HX culture OCN mRNA levels were 2.7 times those of the intact control cultures.

Duplicate cultures were also analyzed by Northern blot to compare steady-state expression levels of OPN and BSP. Levels of OPN expression at day 9 were already 1.5 times intact control levels in the HX cultures (Fig. 5A); BSP levels, however, did not surpass the control until day 12, when they reached a level 3.2 times greater than the intact control cultures (Fig. 5B).
In the HX cultures, expression levels of both of these markers seem to reach peak levels earlier than intact controls, but, by day 16, there is no significant difference between expression levels. Figure 5C is a representative Northern blot depicting steady-state levels of OPN and BSP transcripts in the HX and intact control cultures at days 12 and 14.

**DISCUSSION**

Hypophysectomy is known to result in osteopenia, and the histomorphometric data from these experiments confirm previous conclusions that this osteopenia is, in part, due to a decrease in the osteoblast population on the bone surface (23, 33). Hypophysectomy has also been demonstrated to have a negative effect on hematopoiesis and to result in anemia, leukopenia, thrombocytopenia, and impaired DNA and RNA synthesis in the bone marrow (17). The total number of marrow stromal cells recovered from the HX animals is in parallel with these findings. Total stroma cells recovered from HX animals were significantly less than...
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totals recovered from the intact controls. This study was designed to determine whether or not the reduction in osteogenesis at the bone surface is due to a reduction in the capacity of osteoprogenitors present in the bone marrow to proliferate and develop into mature osteoblasts.

In our initial proliferation studies using a 3-day attachment period, [3H]thymidine incorporation in the HX cultures is significantly greater than in the intact control cultures, but there is no significant difference in cell number at day 8 of culture under nondifferentiating conditions. By day 14, however, the HX group has a significantly greater cell number than the control, demonstrating that the HX committed mesenchymal precursor population, when exposed to an optimal microenvironment in vitro, shows a proliferative capacity that surpasses that of the intact animal. Under differentiating conditions and a 3-day initial attachment period, the HX cultures show greater cell numbers after only 8 days of culture, indicating a possibly greater sensitivity to dexamethasone. When examining total CFU-f under nondifferentiating and differentiating conditions, we again found significantly greater numbers in the HX cultures; when comparing the percentage of total fibroblastic colonies positive for alkaline phosphatase we also found a greater percentage in the HX cultures vs. the control. This implies that the osteoblastic precursors of the HX marrow are a definite contributor to the enhanced proliferative capacity of the marrow stroma. The HX stromal cells also appear to have superior recruitment potential, as evidenced by the greater total number of CFU-f colonies under both conditions. Through visual observation, we also noted that the area of CFU-f and CFU-AP per dish was also greater in the HX cultures, which supports our hypothesis of enhanced proliferative capacity of both mesenchymal precursors and the preosteoblast population.

To investigate the recruitment potential of the two groups we separated the bone marrow stromal cells into adherent and nonadherent fractions as described in MATERIALS AND METHODS and cultured them under nondifferentiating and differentiating conditions. Under nondifferentiating conditions, we discovered that the initial committed mesenchymal precursor population (1-day adherent fraction) of the HX animal appears to be reduced compared with the intact control. However, after 14 days of optimal culture conditions, this fraction of cells from the HX cultures has greater cell numbers, indicating a greater proliferative capacity. The proportion of nonadherent cells in the two groups cannot be deduced in this manner because other factors like recruitment potential contribute to final cell numbers. We also did not measure the proportion of cells in the different fractions and therefore cannot attribute greater cell numbers in the nonadherent fraction of the HX group to greater starting numbers. If our assumption that the initial committed fraction of cells is less in the HX group than when comparing the 5-day adherent fractions, we can say that the committed progenitor population of the HX animal possesses recruitment potential superior to the intact counterparts.

We have also demonstrated that there is a greater induction of the osteoblast phenotype from the noncommitted progenitor population in the marrow stroma by separating the two fractions of progenitor cells and culturing them in differentiation medium. Under these conditions, all fractions from the HX animal showed greater proliferative and differentiation capacities than the intact control cultures. By 14 days of culture in differentiation medium, cell number in the HX cultures was very significantly greater in all fractions, indicating an extreme sensitivity of these cells to differentiating conditions. After examining differentiation markers at the cellular level in the different fractions, we found that AP activity per cell was greater in all fractions of the HX cultures, confirming a greater sensitivity of these cells to differentiating conditions. Because AP activity is not exclusively a marker of osteoblasts, we examined calcium-containing colony-forming units (CFU-Ca) colony numbers and total calcium content as well. These measurements are indicators of mineralization potential. CFU-Ca colony numbers were significantly greater, and calcium content was at significantly increased levels, in all fractions of the HX cultures. These data correlate with the increases in cell number and AP activity of these fractions under differentiating conditions compared with the intact control. Several factors could be at work here. First, the nonadherent and initial adherent progenitors from the pituitary-deficient animal appear to be more sensitive to the inductive properties of the dexamethasone in the medium. Second, the recruitment potential of the adherent precursor population could be augmented as discussed earlier. It has been hypothesized that when the adherent fraction of progenitors is exposed to anabolic stimuli present in the culture medium, they secrete factors such as prostaglandin E2, which has been shown to mediate the transition between nonadherent and adherent preosteoblast cells (24, 25). Because the adherent preosteoblasts from the HX animals have an enhanced response to anabolic stimuli as demonstrated through our proliferation experiments, perhaps they are secreting greater amounts of recruiting factors into the medium and therefore augmenting osteoprogenitor recruitment.

Our examination of molecular markers of differentiation has revealed an earlier progression toward mineralization and enhanced levels of expression in HX cultures. We examined gene expression of two noncollagenous matrix proteins, OPN and BSP. OPN expression has been detected during both the proliferative and differentiation stages of osteoblast development (2, 28, 32), but the role it plays in mineralization has not been clearly defined (32). It has been speculated, however, that its function during the proliferative stage of development is related to the mediation of cell attachment and it conditions the surface of the culture dish in preparation for mineralization (28, 32). As in these other osteoblastic development models, we have also detected this matrix protein during the proliferation stage with considerably higher levels detected in the HX cultures. The second matrix protein of which gene
expression levels were measured, BSP, has been implicated as an influence in the initial formation of mineralized nodules (32). When temporal patterns and peak expression of BSP between the two groups are compared, HX cells not only reach maximal expression earlier than control cells, but peak levels are also greater than those of control cells as well. Using RT-PCR coupled to Southern blot, we also analyzed relative OCN mRNA levels in the two groups. This osteoblastic marker is not only specific to bone but is also highly correlated with the calcification of the ECM (28). By day 12 of culture, there were considerably greater mRNA levels of OCN in the HX cultures, and this difference remained through day 16.

It has been postulated that proliferation is functionally related to the synthesis of the ECM, and the maturation and organization of the ECM contribute to the shutdown of proliferation. This, in turn, promotes the expression of genes that prepare the matrix for mineralization. ECM mineralization or events during the early stages of mineralization are possibly responsible for downregulating genes expressed during ECM maturation and organization (28). Therefore, in this model of osteoblast development, the formation of the ECM is completely related to the stages of differentiation. If this model is applied to the current study, it may help to explain the phenomenon that is occurring in the differentiation of the HX osteoprogenitors. During the first stage, proliferation is elevated, leading to an enhanced deposition of matrix proteins during the ECM maturation stage, as evidenced by increased OPN levels at the end of this stage. This perhaps led to an early shutdown of proliferation and an early and enhanced preparation of the matrix indicated by a temporal shift to the left and greater peak levels of BSP. Ultimately, these events sparked the increased expression levels of OCN and subsequently mineralization. This explanation still leaves us to explain the causative mechanism behind the initial increase in proliferation of the HX osteoblast lineage cells. Why do the in vivo and in vitro results seem in such stark contrast?

The elevated response of the bone marrow stromal cells of the HX animal when placed in vitro could be because of a hypersensitivity of these cells to a factor(s) present in the fetal bovine serum and lacking for an extended period of time in vivo. The most obvious of these is growth hormone (GH), a major systemic growth regulator and mitogenic factor that exerts its effects on bone both directly and indirectly via insulin-like growth factor (IGF)-I (3, 10). Circulating GH interacts with certain receptors located mainly in hepatic tissue, resulting in the stimulation of the synthesis and secretion of IGF-I (1). IGF-I also has paracrine-autocrine properties and is locally secreted by osteoblasts in response to GH (10). Perhaps these osteoprogenitors experience an upregulation of the IGF-I receptor because of the acute lack of GH and, subsequently, the lack of systemic and local IGF-I. It has been demonstrated that human patients with reduced serum levels of IGF-I experienced a compensatory upregulation of lymphocytic IGF-I receptor gene expression in response to low circulating IGF-I levels (11). If this were also true with osteoblasts, it would explain why, when these cells are placed in culture with fetal bovine serum, of which bovine GH is a definite component, they are stimulated to divide at an elevated rate. These receptors initiate the Ras/Raf/MEK/mitogen-activated protein kinase (MAPK) signaling cascade and MAPKs have been indicated as transcriptional regulators of proliferation in osteoblasts (27). This is only one possibility, however, because the HX animal lacks more than just GH. Many other hormones with anabolic properties in bone are also lacking in vivo, such as parathyroid hormone and estrogen. Moreover, GH replacement in HX rats cannot completely restore trabecular bone formation (7), indicating the involvement of other pituitary and/or local growth factors in bone formation.

In conclusion, we have observed that once bone marrow stromal cells from a HX animal are cultured in vitro, they experience an enhanced proliferative and differentiative capacity compared with those of the intact animal. The cellular and molecular mechanisms behind this phenomenon have yet to be elucidated. An understanding of these regulatory mechanisms is critical for gaining insight into the pathogenesis of pituitary deficiency-related osteoporosis and other bone-related diseases, as well as for the development of new treatment regimens.

The authors are grateful to Nancy Ling and Amy Ching-Man Chu for expert technical assistance.

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Received 20 April 1998; accepted in final form 8 September 1998.

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