Osteoblast-like cells of the hypophysectomized rat: a model of aberrant osteoblast development

JO D F. EVANS, JAMES K. YE H, AND JOHN F. ALOIA

Osteoblast-like cells of the hypophysectomized rat: a model of aberrant osteoblast development. Am J Physiol Endocrinol Metab 278: E832–E838, 2000.—In a previous work, we demonstrated that the osteoprogenitors derived from the marrow stroma of the hypophysectomized (HX) rat demonstrate enhanced proliferative and differentiation capacities when placed in an optimal microenvironment. In this study, we sought to investigate the potential of the trabecular osteoblast-like cells of the HX rat. These cells represent a more mature pool of osteoblasts than the progenitors derived from the marrow stroma. We examined all three stages of osteoblast development using trabecular osteoblast-like cells derived from age-matched intact rats as a control. Using thymidine incorporation and cell number as indicators of proliferation, we found that these cells, like the osteoprogenitors derived from the HX rat, demonstrate augmented proliferation when placed in culture. Additionally, type I collagen expression remained at significant levels past the end stages of proliferation, at which point it is expected to be downregulated. Matrix maturation markers, such as alkaline phosphatase activity and bone sialoprotein expression, however, were significantly lower than in the controls. Mineralization potential, as measured by mineralized nodule formation, Ca\(^{2+}\) content, and OPN and OCN expression, was also significantly reduced. Our results have uncovered an aberrant model of osteoblast development in which proliferation is deregulated, resulting in a minimal capacity of these cells to develop into fully differentiated mineralizing osteoblasts.

alkaline phosphatase; mineralization potential; proliferation; differentiation

BONE, A SEEMINGLY FIXED tissue, is continually undergoing regeneration (modeling and remodeling) both during the development and throughout the life of the organism. To maintain this capacity, it must possess precursor cell populations capable of differentiating into functional osteoblasts. It has been long established that cells of the osteogenic lineage are derived from multipotential mesenchymal cells of the bone marrow stroma (1, 2, 15, 18). Results of studies performed on animals have confirmed this hypothesis, and it has been shown that renewal of the osteoblast population on the bone surface is dependent on the recruitment, proliferation, and differentiation of this precursor pool.

More recently, it has been demonstrated that there is a more committed precursor pool located on the trabecular surface (11). Culture systems have been established in an effort to characterize both the osteoprogenitors present in the marrow stroma and the more committed preosteoblast derived from the primary spongiosa (17, 28).

Three principle stages have been delineated for these primary-derived osteoblasts: proliferation, extracellular matrix (ECM) maturation, and ECM mineralization (22–24). As the osteoblast progresses through these developmental stages, there is a sequential expression of cell growth and tissue-specific genes. For example, during the proliferative stage, genes associated with the activation of proliferation such as c-fos are expressed together with cell cycle progression genes like histone. Also during this period, genes associated with the regulation of ECM biosynthesis such as transforming growth factor-β and type I collagen (COLL-I) are highly expressed (22–24). Alkaline phosphatase (AP) and bone sialoprotein (BSP) expression are relatively high during the matrix maturation stage. These genes are associated with the maturation and organization of the bone ECM and prepare the matrix for mineralization. During the third stage, mineralization, osteopontin (OPN) and osteocalcin (OCN) are maximally expressed (22–24).

A reciprocal and functionally coupled relationship exists between cell growth and differentiation-related gene expression during osteoblast development. It has been postulated that proliferation is functionally related to the generation of a bone-specific ECM, and the organization and maturation of the ECM contributes to the shutdown of proliferation. This shutdown in turn promotes the expression of genes involved in preparing the matrix for mineralization. Mineralization or early events during mineralization are possibly responsible for the downregulation of the ECM maturation-related genes. Two restriction-transition points have been identified during the development of the osteoblast. These points have been experimentally established and functionally defined as points at which developmental expression of genes are halted until additional cellular signals are received. The first transition point is the completion of the proliferative period when cell cycle and growth-related genes are downregulated and genes involved in ECM maturation are expressed. The onset of ECM mineralization represents the second transition point. Understanding of the signaling mechanisms

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operating at these points is critical for gaining insight into the relationship between proliferation and differentiation in the osteoblast cell.

The hypophysectomized (HX) rat has traditionally been used as a model to study the effects of pituitary hormone deficiency on skeletal growth. Systemically, HX results in a suppression of weight gain, a reduction in lean mass, and decreased skeletal growth (5, 6, 29). One of the most striking effects demonstrated in vivo is a cessation of longitudinal growth (12, 27). Studies focusing on de novo bone differentiation have demonstrated that HX results in a marked reduction and delay in osteogenesis reflected by an inhibition of mesenchymal cell proliferation. In addition, these studies have also revealed that the quality of newly formed bone is also deficient (19, 26). Furthermore, histomorphometric studies performed in our laboratory have revealed a reduction in the osteoblast surface of HX rats.

Our laboratory has recently completed an investigation of the osteogenic potential of the bone marrow stroma of the HX animal. We have found that, once these cells are placed in an optimal in vitro environment, they display an enhanced proliferative and differentiative capacity (30). The elevated response of these cells could be due to a hypersensitivity of these cells to factor(s) present in the FBS and lacking for an extended period of time in vivo. These results are in such contrast to results obtained from in vivo histomorphometric studies that we were prompted to investigate further the osteogenic potential of the HX precursor pools. Therefore, this study was undertaken to determine whether the more mature osteoblast progenitors of the trabecular surface show the same enhanced capacities in vitro as those derived from the bone marrow stroma. We examined all three stages of osteoblast development using cell number as an indicator of proliferation and COLL-I expression as a marker of the final stage of proliferation when matrix biosynthesis begins. AP activity and BSP expression were used as markers of matrix maturation. Finally, nodule formation, Ca\(^{2+}\) content, and OPN and OCN expression were used as indicators of mineralization.

**MATERIALS AND METHODS**

**Animals**

Age-matched HX and intact control female 8-wk-old Sprague-Dawley rats were purchased from Hilltop, where the hypophysectomy surgeries were performed. Upon 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.

**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 epiphyseal cartilage, and the growth plate was scraped away with a scalpel and discarded. Trabecular bone was then mechanically removed from the proximal tibiae and distal femurs. These bone fragments were then minced into 1- to 2-mm pieces and washed several times in Ca\(^{2+}\)-free Hanks’ balanced salt solution (HBSS; Life Technologies, Grand Island, NY) to remove any contaminating marrow fragments. The bone fragments were then digested with a 2-mg/ml collagenase type II/HBSS solution for 20–30 min, after which the supernatant was removed, and the fragments were washed twice with HBSS. The trabecular bone fragments were then plated with α-MEM supplemented with 10% FBS, 50 µg/ml ascorbic acid, 2 mM L-glutamine, and antibiotic/antimycotic in 100-mm culture dishes, and surface cells were allowed to grow out from the bone. Medium was changed every 2–3 days, and after 7–9 days, the cells reached ~75% confluence and were trypsinized, filtered through a 70-µm filter, and counted using trypan blue exclusion and hemocytometry. Harvested cells were then plated according to experimental objectives either in the above medium with the addition of 10 mM β-glycerol phosphate (differentiation medium) or in differentiation medium supplemented with 10\(^{-8}\) M dexamethasone. Harvested cells were then plated according to experimental objectives.

**AP Activity**

Cells were initially plated at a density of 5 \times 10\(^3\)/well of a 12-well plate. On days 8, 11, 13, and 15 of culture, the cells were washed with 1× PBS and fixed with ice-cold 95% ethanol. The cells were then washed with dH\(_2\)O and allowed to equilibrate in 20 mM bicarbonate buffer, pH 8.8, followed by a 5- to 10-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 by spectrophotometry at 405 nm, and the results were expressed as nanomoles per minute per 10\(^6\) cells. Cell number for the AP cultures was obtained by the method of Currie (8). Briefly, after the AP activity of the cells was measured, the cells were fixed again in 10% phosphate-buffered Formalin for further mechanical stability. They were then washed one time with tap water and two times with borate buffer (10 mM, pH 8.8), stained with 1% methylene blue in borate buffer for 15 min, and then washed several times with tap water followed by borate buffer. 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.

**Mineralization Assay**

Cells were initially plated at ~10\(^{3}\)/cm\(^2\). On day 18, cultures were washed with PBS and fixed with 10% phosphate-buffered Formalin. Mineralized nodules were histochemically stained using alizarin red, pH 6.2 (20, 30).

**Calcium**

Cells were initially plated at ~10\(^{4}\)/cm\(^2\). On the days stated in text, cells were fixed with 10% phosphate-buffered Formalin for 2 h and then were rinsed with dH\(_2\)O. Calcium in the cell layers was extracted using 1% HCl, and calcium content was measured using atomic absorbance.

**Northern Analysis**

Cultures were initially plated at 5 \times 10\(^6\)/60-mm dish, and on the days stated in the text, total cellular RNA was...
extracted from cultures using Trizol Reagent (Life Technologies) following the recommendations of the manufacturer. Aliquots (10 µg) were fractionated on 1.2% agarose-formaldehyde gels and then transferred to a Biodyne B membrane (Life Technologies) with 20× saline sodium citrate (SSC) overnight. Membranes were prehybridized in Hybrisol I (Oncor, Gaithersburg, MD) and 0.5 mg/ml sonicated salmon sperm DNA at 42°C for 2 h. The following DNA probes were used: a 1.0-kb mouse OPN EcoRI fragment, a 1.0-kb mouse BSP EcoRI fragment, a 450-bp EcoRI/PstI fragment (generous gifts of Dr. David Rowe, University of Connecticut, Farmington, CT), a 2.1-kb COLL-I cDNA excised from an Hf32 construct purchased from the ATCC (no. 61484), and a 1.2-kb EcoRI human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) fragment (Clonetech, Palo Alto, CA). Probes were labeled with [32P]dCTP (NEN, Boston, MA) using 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, 1 room temperature wash with 0.2× SSC-0.1% SDS for 15 min, and a final wash with 0.2× SSC-0.1% SDS at 42°C for 15 min. The membranes were 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 using the SigmaGel computer program (Jandel Scientific Software, San Rafael, CA) and a Hewlett Packard (HP) Desk Scan II scanner. Expression levels were normalized to GAPDH expression.

RT-PCR for Semiquantitation of OCN Expression

Cultures were extracted for RNA as stated above. Total RNA (1 µg) was used in each reaction and was first treated with RNase-free DNase I (Life Technologies) and then reverse transcribed under conditions previously described (30). Aliquots (10 µl) of the reverse transcription reaction were used in the simultaneous PCR reactions for rat OCN and rat GAPDH. PCR conditions were as previously described (30). Quantitation of PCR products and confirmation of sequence identity were performed by Southern blot analysis followed by chemiluminescent detection, as previously described (30). Band intensities were measured using the SigmaGel computer program (Jandel Scientific Software) and a HP Desk Scan II scanner. OCN expression was normalized to GAPDH.

RESULTS

Proliferation

Cell number. The HX cultures had a higher cell number under both culture conditions at all time points measured (Fig. 1). In the differentiation medium, cell number in the control cultures plateaued at ~11 days, where in the HX cultures cell number increased up until the last day measured, day 15. Similarly, the cell number in the control cultures leveled off by day 11 in the dexamethasone-supplemented medium, and the HX cultures continued to expand until day 13 when they reached a plateau. Our [3H]thymidine measurements confirm these results (not shown).

Differentiation

AP activity. In the differentiation medium, AP activity per cell in the control group remained level at all days measured, whereas in the HX group AP activity was at its highest at day 8 and continued to decline thereafter (Fig. 2). In this medium, the HX group went from 70% of control values at day 8 to 30% at day 15. The control cells reached peak AP activity at day 11 in the dexamethasone medium and then declined. HX cultures followed a similar pattern but at a significantly lower level of activity.

Mineralization. Nodule numbers (Fig. 3) in the control cultures were significantly greater than in the HX cultures (49.5 ± 13.38 control vs. 8.5 ± 2.08). Calcium content (Table 1) in the control cultures was also significantly greater than in the HX group.

Molecular markers. When comparing differences in COLL-I expression between groups (Fig. 4, A and B), we found that the expected downregulation started at day 20 in the control cultures and reached minimal levels by day 27. The HX cultures demonstrated no appreciable decrease in COLL-I expression, and the

![Graph](http://ajpendo.physiology.org/ by 10.220.32.246 on September 8, 2017)
levels remained high through day 27. In cultures treated with dexamethasone, the controls reached peak BSP expression by day 10 (Fig. 4, C and D) and then began to decrease by day 14. The HX cultures reached a slight peak at day 14, significantly lower than control, and remained at this level through day 21. In control cultures grown in differentiation medium, a marked increase in OPN mRNA levels occurred at day 20, and peak levels were reached by day 25 (Fig. 4, A and B). HX cultures demonstrated slight peaks at days 11 and 25. Peak OPN expression in the HX cultures during the mineralization phase was significantly lower than the control. With dexamethasone in the medium, OPN expression in control cultures peaked at day 12 and began to decline by day 18 (Fig. 4, C and D). On the other hand, HX cultures do not reach peak expression levels until day 14, and these are 24% below peak control levels. OCN mRNA levels in cultures not treated with dexamethasone are not detectable by Northern analysis in our laboratory. Therefore, we used a more sensitive method of detection, RT-PCR, to semiquantitate differences in expression levels between groups. With this method, we were able to detect OCN mRNA at appreciable levels by day 15 in these cultures (Fig. 4, A and B). OCN expression in control cultures reached a peak at day 25, and this level remained high through day 27. HX cultures reached a slight peak at day 25 but at a level 48% below the control. The same method of detection was used for the cultures treated with dexamethasone. OCN mRNA levels were detectable by day 10 of culture with dexamethasone using this method (Fig. 4, C and D). Under these conditions, control cultures reached peak levels by day 12. On the other hand, HX cultures did not peak and remained at a level ~40% below peak expression in the control. Northern analysis of these cultures for OCN showed similar results (data not shown).

**DISCUSSION**

Unlike our results with the bone marrow of the HX animals, where we found both an enhancement in proliferation and differentiation, the trabecular osteoblast-like cells, a more mature pool of cells, showed an increased proliferation response to an in vitro environment; however, their capacity to differentiate was greatly diminished. The results from the proliferation and matrix maturation studies, if taken together, indicate a disruption in the mechanism(s) active at the first restriction-transition point of development in these HX cells, and alterations observed at the second point appear contingent upon the disruption occurring at the first.

Under both culture conditions, the trabeculae-derived cells of the HX rat showed a significant increase in proliferation rate, as evidenced through both cell number and thymidine incorporation assays, compared with their age-matched intact counterparts. Under typical growth conditions, proliferation in osteoblast cultures levels off at the point where the first stages of differentiation begin (22–24), as seen in our control cultures. Cell numbers in the HX cultures, however, continued to expand past the expected transition into the differentiation stages of development. Furthermore, COLL-I levels in the HX cultures remained at significant levels until the last day of culture when grown in differentiation medium. It has been suggested that COLL-I synthesis is functionally coupled to the downregulation of proliferation and is associated with the ECM biosynthesis (14, 22, 23). This reciprocal relationship could not be demonstrated in the HX cultures. From this, we have concluded that the signaling mechanism responsive to accumulation of matrix protein is impaired, and accordingly there is a failure to shut down proliferation. Glucocorticoids inhibit collagen synthesis in vitro (7); therefore, COLL-I is not present at detectable levels when these cells are propagated with dexamethasone in the medium. In contrast to the proliferation results, markers of the matrix maturation and organization stage, AP activity and BSP expression were significantly below control levels.

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**Table 1. Calcium content in intact vs. hypophysectomized cultures**

<table>
<thead>
<tr>
<th>Day of Culture</th>
<th>Calcium Content, mg/dl</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>15</td>
<td>0.68 ± 0.27</td>
</tr>
<tr>
<td>19</td>
<td>8.13 ± 2.62</td>
</tr>
</tbody>
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Values are expressed as means ± SD of 6 replicate cultures/animal. HX, hypophysectomized. Cultures were grown in dexamethasone-supplemented medium. Data are representative of 2 experiments yielding similar results. *P ≤ 0.01 as determined by the Student’s t-test.
Fig. 4. A: representative Northern blot depicting type I collagen (COLL-I) and osteopontin (OP) expression in the cultures grown in differentiation medium along with a representative Southern blot depicting osteocalcin (OC) expression as semiquantitated by RT-PCR. Nos. indicate the no. of days in culture. B: densitometric analysis of Northern and Southern blots in A. Open bars, control cultures; filled bars, HX cultures. Values are means ± SD of 2 separate blots. **P ≤ 0.01 and *P ≤ 0.05 as determined by the Student’s t-test. C: representative Northern blot depicting bone sialoprotein (BSP) and OP expression in cultures grown in differentiation medium with the addition of dexamethasone along with a representative Southern blot depicting OC expression as semiquantitated by RT-PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. D: densitometric analysis of Northern and Southern blots in C. Open bars, control cultures; filled bars, HX cultures. Values are means ± SD of 2 separate blots. *P ≤ 0.05 as determined by Student’s t-test.
in the HX cells. These results taken together with the proliferation results further indicate that the reciprocal and functionally coupled relationship between the organization of the ECM and the downregulation of proliferation is significantly impaired in the HX cells.

In our investigation of second-stage markers, BSP mRNA did not reach levels detectable by Northern blot analysis without dexamethasone. Dexamethasone is a strong inducer of BSP, a protein specific to all mineralizing tissues, which is expressed at peak levels coincident with the formation of the ECM. Constitutive levels of this protein under standard differentiation conditions are likely present but perhaps are not detectable under our experimental conditions. Also, AP activity does not change over time in the control cells grown without dexamethasone, as might be expected. This population of osteoblast-like cells has not been as extensively studied as those derived from marrow or fetal calvariae. This may be a typical pattern of development for these cells in culture. It is possible that we began measuring AP activity after it had reached peak levels. A study characterizing osteoblastic cells from long bone demonstrated that these cells reached peak AP activity very early in culture, equivalent to our day 7, and declined thereafter, remaining at background levels for the remainder of the culture period.

When we went on to compare and contrast the mineralization phase, this disruption in the pattern of osteoblastic development of the HX cells continued. Results obtained from the measurement of both cellular and molecular markers of differentiation lead us to conclude that the mechanism(s) active at the second transition-restriction point are also disrupted in the HX cells. Under accelerated differentiation conditions, both calcium content and mineralized nodule formation are significantly less in the HX cultures, and, by visual observation, nodule formation in the HX cultures grown without dexamethasone was sparse and failed to mineralize, unlike their intact control counterparts (data not shown). Additionally, OPN and OCN expression in the HX cultures grown in medium without dexamethasone never reached the strong peaks observed in the control cultures and remained at significantly lower levels throughout the experiment. A minimal amount of mineralization did occur in the HX cultures grown in the dexamethasone medium. The addition of dexamethasone to the medium perhaps altered the microenvironment enough to allow a slight and delayed transition into the mineralization phase in these cultures.

Both the osteoprogenitors (bone marrow stroma) and the more mature osteoblast-like cells of the trabeculae had been exposed to the same deficient environment in vivo, the absence of hypophyseal hormones, for the same period of time, and both cell types experienced an elevation in proliferation rate when placed in culture. What mechanism then is responsible for the failure of the HX trabeculae-derived cells to differentiate properly? The initial hyperproliferative response of both cell types is likely due to changes in the factors present in the microenvironment. Alterations in the growth hormone (GH)/insulin-like growth factor (IGF) 1 axis in the HX animal are likely a significant contributor to these changes.

GH is a major systemic growth regulator and mitogenic factor that exerts its effects on bone both directly and indirectly via IGF-I (3, 9). GH deficiency in the HX animal leads to low circulating levels of IGF-I and perhaps to an upregulation of the IGF-I receptor. Patients with reduced serum levels of IGF-I have been shown to experience a compensatory upregulation of lymphocytic IGF-I receptor transcription (10). IGF are important autocrine and paracrine mitogens in bone and are usually found to be associated with IGF-binding proteins (IGFBPs), which regulate many of their biological actions. IGFBP-1 has been shown to inhibit the anabolic effects of IGF-I, and its hepatic transcription is rapidly downregulated by GH (13). GH deficiency has resulted in markedly elevated IGFBP-1 levels (16). IGFBP-3, on the other hand, has been shown to enhance IGF-I activity (7), and its maximal expression is seen in the more mature osteoblast (4). In vivo, IGFBP-3 expression is GH dependent. Low circulating and local IGF-I levels increased IGFBP-1, and decreased IGFBP-3 levels could in part be responsible for the reduction in osteogenesis seen at the bone surface in the HX animal. However, when these cells are placed in culture, the inhibitory effect of high IGFBP-1 levels is abolished, rat osteoblasts do not express IGFBP-1 in vitro (4), and locally produced IGF is free to exert its anabolic effect. This together with the upregulated IGF-I receptor could be responsible for the enhanced proliferation in culture. Furthermore, the bovine GH (BGH) in the serum could be inducing the production of IGFBP-3 in the more mature trabecular cultures, further enhancing the IGF-I proliferative response.

It is the presence of the IGFBP-3 in the trabecular cultures that could be responsible for preventing these cells from achieving their differentiation potential. This enhancer of the IGF-I pathway could be responsible for overstimulating this proliferation pathway and thereby disrupting the mechanism responsible for the transition to the differentiation stages of development. This can be substantiated by the fact that, when the HX cells are grown in the presence of dexamethasone, they are capable of limited differentiation. Dexamethasone is a known inhibitor of osteoblast proliferation in vitro, and it has been postulated that these effects are mediated through altering the expression of IGFBPs. It has been specifically demonstrated to decrease IGFBP-3 mRNA levels in human osteoblast cultures by 25% (7). It is perhaps this decrease that leads to the minimal differentiation potential reached by the HX cultures grown with dexamethasone. Further studies investigating the IGF-I response pathway and the interactions of IGFBPs are necessary to confirm these hypotheses.

In conclusion, through the investigation of trabecular osteoblast-like cells of the HX, we have uncovered a model of aberrant progression along the osteoblastic lineage. With the employment of various molecular techniques, this model can be instrumental in the investigation of the intricate relationship between pro-
life and differentiation in osteoblastic development and may provide insight into the mechanisms active at the transition points along this pathway.

Address for reprint requests and other correspondence: J. Evans, Metabolism Laboratory, Dept. of Medicine, Winthrop-Univ. Hospital, Mineola, NY 11501 (E-mail: jevans@winthrop.org).

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