Influence of growth hormone on bone marrow adipogenesis in hypophysectomized rats

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In conclusion, growth hormone administration to hypophysectomized rat has been used as a model to study the effects of growth hormone deficiency on bone. Here, we have investigated the influence of growth hormone administration to hypophysectomized rats (HX) for 6 wk on accumulation of triglycerides in bone marrow and on the differentiation of primary marrow stromal cells into adipocytes under in vitro conditions. We found that hypophysectomy significantly increased triglyceride concentration in bone marrow, which was attenuated by growth hormone administration. Primary bone marrow stromal cells derived from HX rats also had more adipocytes at confluence compared with growth hormone-treated hypophysectomized (GH) rats. When stimulated with 3-isobutyl-1-methylxanthine plus dexamethasone (IBMX-Dex), preadipocyte colony counts increased more significantly in GH rats. Markers of adipocyte differentiation were higher in HX than in control or GH rats at confluence. However, after stimulation with IBMX-Dex, increased expression of markers was seen in GH compared with HX rats. In conclusion, growth hormone administration to hypophysectomized rats attenuated triglyceride accumulation in bone marrow and inhibited the differentiation of stromal cells into adipocytes in vitro.

bone marrow; triglycerides; adipocytes

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tion factors to appear in the committed adipocyte and is important in the control and synthesis of other genes, transcription factors, enzymes, transport proteins, and hormones required for the metabolic activities of the mature adipocyte (15). Lipoprotein lipase (LPL) is an enzyme that appears in the early adipocyte and is responsible for the accumulation of triglycerides in the fat cells. Other genes controlled by these early transcription factors include adipin and leptin, intermediate and late markers, respectively, of adipocyte differentiation (26).

In recent studies, it was observed that the in vitro differentiation of primary marrow stromal cells derived from hypophysectomized rats into osteoblasts is enhanced compared with controls (30). Here, we have examined the effects of in vivo administration of growth hormone to hypophysectomized rats on the accumulation of triglycerides in femur as well as in vitro induction of adipogenesis in the bone marrow stroma. The rationale for the study is that the lack of growth hormone in the hypophysectomized rat in vivo will lead to enhanced differentiation of stromal cells into adipocytes in vivo, with fewer stromal cells available for in vitro adipogenic induction. It is expected that treatment of hypophysectomized rats with growth hormone will inhibit the commitment of bone marrow stromal cells into adipogenesis in vivo, thereby making more uncommitted stromal cells available for induction of adipogenesis under in vitro conditions.

MATERIALS AND METHODS

Animal care. Age-matched intact control (CT) and hypophysectomized female 8-wk-old Sprague-Dawley rats were purchased from Hilltop Animal Care (Pittsburgh, PA), where the hypophysectomies were performed. Upon arrival 4 days postoperatively, the animals were divided into three groups of six each for the study design: age-matched intact CT, hypophysectomized control (HX), and hypophysectomized plus growth hormone (GH). All animals were allowed access to standard pelleted rat chow (Rodent Laboratory Chow 5001; Winthrop-University Hospital approved the animal protocol. Brieﬂy, 100-μl aliquots of the supernatant samples from each femur were pipetted in triplicate into glass tubes, and 500 μl of hexane were added. After a 30-min incubation at room temperature, samples were transferred into polystyrene tubes and immediately dried under nitrogen. Oil red-O was added to each tube, incubated for 20 min, and decanted. Tubes were washed three times with distilled water and dried. Oil red-O was then determined from a standard curve, which was derived by measuring the optical density of known concentrations of stained triolein at 510 nm.

Northern blot analysis. RNA samples were isolated from 12-well culture dishes at the following time points: confu-
ence and days 1 and 7 post-IBMX-Dex treatment. Cells were washed once with PBS, and total cellular RNA was extracted using TRIzol reagent (GIBCO-BRL) according to the manufacturer’s instructions. The RNA samples were quantitated and stored at −80°C until ready for use. Aliquots (10 μg) of RNA samples were fractionated on 1.2% agarose-formaldehyde gels and transferred onto Biodyne B membranes (Life Technologies) overnight in 20× standard sodium citrate (SSC). Membranes were baked at 80°C for 1 h and prehybridized in Hybrisol-1 (Integen, Gaithersburg, MD) for ≥2 h at 45°C. The following cDNA probes were used for hybridization: a 261-bp adipin fragment, a 640-bp LPL fragment, and a 1.2-kb EcoRI human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment (Clontech, Palo Alto, CA). For adipin and LPL, the cDNA was cloned into the PCR-Trap Cloning System (GeneHunter, Nashville, TN), and sequences were verified. Probes were labeled with [32P]dCTP (NEN, Boston, MA) using PCR. Hybridization was carried out overnight at 45°C, followed by one or two washes at room temperature with 2× SSC-0.1% SDS for 15 min each. The membranes were exposed to Fuji NIF film for variable periods of time with intensifying screens at −80°C.

**RT-PCR and Southern blot analysis.** RNA (1 μg) was initially treated with DNAse 1, reverse transcribed, and subjected to amplification by PCR as previously reported (30). Diluted cDNA template from the RT reaction (5 μl) was used (leptin 1:5, PPARγ 1:10) for PCR. The reaction mixture consisted of 20 mM Tris·HCl (pH 8.0), 50 mM KCl, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1 mM MgCl₂, 1.23 U of Taq DNA polymerase (Life Technologies), and 50 pmol each of forward and reverse primers. Cycle lengths were 20 for leptin and 25 for PPARγ, and these were determined to be within the linear range for the respective message.

The PCR products were fractionated on 1.2% agarose gels which were then denatured, neutralized, and transferred to Biodyne B membrane (Life Technologies) with 20× SSC overnight. Membranes were baked at 80°C for 1 h and then prehybridized in Hybrisol-1 (Integen) for ≥1 h. Hybridization was carried out overnight at 45°C by adding [32P]dCTP-labeled probes. After hybridization, membranes were washed twice at room temperature in 2× SSC-0.1% SDS for 15 min each. Detection was done by exposure to Kodak X-OMAT scientific imaging film for various periods of time.

The sequences of the primers used for PCR analysis were as follows: PPARγ2, left CAT TTT TCA AGG GTG CCA GT, right TTA TTC ATC AGG GAG GCC AG; Leptin, left TGA CAC CAA AAC CCT CAT CA, right CAT TCA GGG CTA AGG TCC AA. The sequences for the GAPDH primers have been previously reported (30).

**Semiquantitative analysis.** Blots were scanned with an HP Desk II scanner for band intensities, which were subsequently analyzed with the SigmaGel computer program. GAPDH expression was used to normalize the data for the PCR, and the intensity of the 28S band was used to normalize the Northern blot analysis data.

**Results**

In vivo adipogenesis. The weights of the rats in the various groups are shown in Table 1. The average weight of the hypophysectomized rats at the start of the study, 1 wk after surgery, was 192.5 ± 5.7 g, whereas that of the age-matched CT rats was 221.3 ± 7.3 g. At the end of the 6-wk injection period, the average weight of the HX group was, as expected, not significantly different from the starting weight (204.6 ± 10.6 vs. 192.5 ± 5.7 g). However, there was a significant weight increase of 23.2 and 37.4% in the CT and HX groups, respectively. The final weight of the GH rats did not differ from that of the CT rats.

To study the effect of in vivo growth hormone treatment on bone marrow adipogenesis, the triglyceride content of bone marrow extracted from the femurs and the number of preadipocytes were determined as well (Fig. 1). The number of preadipocytes in the smear expressed as a percentage of oil red-O-positive cells was significantly lower and the triglyceride content of femur was significantly higher in the HX group than that of the CT group. In vivo growth hormone treat-

**Table 1. Weight of rats**

<table>
<thead>
<tr>
<th></th>
<th>Start</th>
<th>End</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>231.3 ± 7.3</td>
<td>272.7 ± 19.6</td>
<td>23.2</td>
</tr>
<tr>
<td>HX</td>
<td>192.5 ± 5.7</td>
<td>204.6 ± 10.6</td>
<td>6.3</td>
</tr>
<tr>
<td>GH</td>
<td>195.5 ± 6.8</td>
<td>265.7 ± 29.7</td>
<td>37.4</td>
</tr>
</tbody>
</table>

Values are means ± SE in grams. CT, control group; HX, hypophysectomized group; GH, growth hormone-treated, hypophysectomized group.

**Statistical analysis.** The two-tailed Student’s t-test was used to compare differences in response between the groups to both in vivo and in vitro conditions. A P value of <0.05 was considered as significant.

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ment of hypophysectomized rats resulted in significant increase in preadipocytes but a significant reduction of triglyceride content of femur. 

**In vitro adipogenesis.** Because hypophysectomy is known to cause increased adipogenesis in vivo, the influence of in vivo treatment of GH rats on the ability of primary marrow stromal cells to differentiate into adipocytes was studied under cell culture conditions. The numbers of adipocytes and preadipocytes were counted, and expression of markers of adipocyte differentiation (PPARγ2, LPL, adipsin, and leptin) was measured at confluence and on days 4 and 10 postconfluence under basal conditions and after stimulation with 450 μM IBMX and 1 μM Dex for 3 days. In pilot studies involving three different concentration levels of IBMX-Dex, the greatest response in terms of adipocyte marker induction was seen with 1 μM Dex and 450 μM IBMX (data not shown).

**Mature adipocytes.** Figure 2A shows expression of the mature adipocyte phenotype when primary marrow stromal cells were followed in culture until and up to 10 days after confluence. At confluence, the adipocyte count was significantly higher in the HX group but lower in the GH group compared with CT. The number of adipocytes decreased in all groups by day 4 postconfluence and persisted to day 10. Treatment of confluent cells with IBMX-Dex did not affect the mature adipocyte numbers (Fig. 2B).

**Preadipocyte colonies.** The number of preadipocyte colonies was highest in the GH group at confluence and lowest in the CT group. In nonstimulated cells, there was no significant change in the colony counts at 4 days postconfluence compared with confluence levels, but by 10 days postconfluence there was a slight increase in CT and HX rats (Fig. 2C). Stimulation of confluent cells with IBMX-Dex resulted in a significant increase in preadipocyte colony counts in all three groups at day 4. The GH group had a significantly higher preadipocyte colony count than the HX group at day 4 that persisted to day 10, albeit at a lower level (Fig. 2D).

**Triglyceride extraction.** Oil red-O was extracted from culture wells to determine the accumulation of triglycerides under in vitro conditions (Fig. 3). There were significantly more triglycerides in GH than in CT (0.23 ± 0.06 vs. 0.16 ± 0.03, P < 0.02) or HX rats (0.23 ± 0.06 vs. 0.17 ± 0.02, P < 0.01) at confluence.
Without stimulation with induction agents, the triglyceride content in all three groups decreased over time. When confluent cells were stimulated with IBMX-Dex, there was a slight but not significant increase in optical density of 6.1 and 19.8% in CT and HX rats, respectively, at day 4. There was an increase of 41% in optical density of the GH group, which was significantly different from that of both CT and HX groups (P < 0.01). By day 10 postconfluence, the increase in the GH group persisted but lower at 21%, which was still significantly different from HX (P < 0.001).

**Markers of adipocyte differentiation.** The expression of mRNA for all four markers was detected in the three groups when primary rat marrow stromal cells were grown to confluence and then followed for up to 10 days postconfluence (Fig. 4).

After confluence, cells were followed in culture for up to 10 days with or without stimulation with IBMX-Dex, and the expression of markers of adipocyte differentiation was determined. Under basal conditions, the expression of leptin mRNA decreased in HX and CT rats by day 4 postconfluence, with almost undetectable levels in HX rats by day 10 postconfluence (Fig. 5A). In the GH group, expression of leptin mRNA increased by day 4 postconfluence and then decreased to confluence level by day 10. After treatment of confluent cells with IBMX-Dex, the decrease in expression of leptin at day 4 postconfluence (day 1 posttreatment) in HX rats was accelerated. However, at day 10 postconfluence, increased expression occurred in the HX group. There was no change in leptin mRNA expression in the GH group at day 10 after treatment with IBMX-Dex compared with the HX rats.

Stimulation with IBMX-Dex caused a decrease in expression of adipsin in HX rats at day 4, but an increased expression was observed in GH rats compared with nonstimulated cells (Fig. 5B). There was a significant increase in adipsin expression in all three groups at 10 days following stimulation with IBMX-Dex.

The expression of LPL mRNA was increased in stimulated cells from the CT and GH groups by day 4 but decreased in similarly treated cells from the HX group. By day 10, however, there was no difference in the
expression of LPL mRNA between cells from all three groups whether stimulated with IBMX-Dex or not (Fig. 5C).

At day 4 postconfluence, the expression of mRNA for PPARγ2 was similar in stimulated and nonstimulated CT and GH groups (Fig. 5D). However, there was a decreased expression of mRNA in the stimulated HX group. By day 10, whereas significant increases were observed in the CT and GH groups following IBMX-Dex stimulation, a significant decrease occurred in the HX group, leading to almost undetectable levels.

**DISCUSSION**

Hypophysectomy resulted in increased bone marrow adipogenesis and fat accumulation compared with the CT rats, as demonstrated by the increased triglyceride content of bone marrow. Treatment of hypophysectomized rats with growth hormone reversed these changes. The increased adipogenesis in HX rats continued in vitro when primary bone marrow stromal cells were allowed to grow to confluence under basal conditions. This was reflected not only in the higher number of adipocytes in HX rats but also in increased expression of markers of adipocyte maturation: PPARγ2, adipin, and leptin. The data suggest that growth hormone inhibits differentiation of marrow stromal cells into adipocytes and that its absence in HX rats allowed the pluripotent bone marrow stromal cells to differentiate along the adipocyte pathway leading to increased adipogenesis. Furthermore, the effects due to lack of growth hormone in HX rats in vivo persisted in vitro, as there were significantly fewer adipocytes in GH than in HX or CT rats at confluence. This finding is supported by that of Desle et al. (6) as well as Hansen et al. (12). In studies using primary preadipocytes directly obtained from rats, it was reported that growth hormone stimulated proliferation and had a potent inhibitory effect on differentiation of preadipocytes into mature adipocytes. However, in other studies in which preadipocyte cell lines such as 3T3-F422A, 3T3-L1, and Ob1771 were used, growth hormone was shown to inhibit proliferation of preadipocytes and promote their differentiation into mature adipocytes (28). Thus it appears that the role of growth hormone in adipocyte differentiation varies depending on the type of cells used, being adipogenic in preadipocyte cell lines and antiadipogenic in both primary marrow stromal cells and primary preadipocytes.

Because osteoblasts and adipocytes are both derived from the same mesenchymal progenitor cells, stimulation of adipogenesis and accumulation of fat in bone marrow would be expected to lead to decreased bone formation and osteopenia/osteoporosis in the long term. Previous studies have demonstrated that hypophysectomy leads to a decrease in bone mineral density in rats and that treatment with growth hormone partially reverses this syndrome (5). Also, in humans, growth hormone deficiency is known to cause osteopenia or osteoporosis, which are significantly improved after initiation of growth hormone therapy (14). Consistent with these findings, administration of growth hormone to hypophysectomized rats in our study reduced the fat content of the femur toward levels found in the CT rats. A combination of factors could account for the reduction of bone marrow fat content of the femur under the influence of growth hormone. In part, this could be due to the inhibition of stromal cell differentiation into adipocytes while at the same time stimulating differentiation along the osteoblast pathway (30). The direct metabolic effects of growth hormone on adipocytes also could play a role in the decrease in marrow fat content of femur. In human adipose tissue, growth hormone suppresses lipogenesis by inhibiting LPL (21, 25). In addition, Dietz and Schwartz (7) reported the activation of hormone-sensitive lipase by growth hormone in 3T3-F422A preadipocytes, leading to increased lipolysis. Together, these studies suggest that growth hormone can act via multiple mechanisms to reduce the accumulation of fat in the bone marrow of femur.

In culture, adipocyte counts progressively decreased in all three groups of rats after cells became confluent, with a more rapid decline noted in HX rats. Several reasons could account for this. First, mature adipocytes are less adherent and more buoyant than preadipocytes, owing to their content of low-density lipid (8). Thus it is possible that some are mechanically removed during medium change. Second, mature adipocytes can undergo apoptosis when cultured in vitro due to various factors such as serum deprivation and heat injury (23). Other agents, including tumor necrosis factor-α and all-trans retinoic acid, also induce apoptosis of human and rat adipocytes, respectively (13, 22). In contrast, other studies have reported that, although the 3T3-L1 preadipocytes undergo apoptosis when subjected to growth factor deprivation, they become resistant after differentiation into adipocytes. However, Zhang et al. (31) reported that apoptosis was observed in 10–15% of mature human adipocytes cultured in normal, serum-containing media with no apoptosis-inducing agents. Our data suggest that apoptosis of mature adipocytes might have occurred after growing to confluence in normal media and that the lack of growth hormone in vivo may have accelerated the process in the HX group. We have not, however, been able to establish this fact conclusively, and further studies are needed.

Dex used in combination with IBMX is a standard protocol for in vitro induction of differentiation in preadipocyte cell lines as well as primary bone marrow stromal cells into mature adipocytes (15, 26). Confluent cells are treated for 3 days with IBMX-Dex and withdrawn. We did not observe any increase in adipocyte counts when confluent primary marrow stromal cells from rats were stimulated with IBMX-Dex. In fact, when followed for up to 7 days after removal of IBMX-Dex, the adipocyte counts continued the downward trend noted previously. In contrast, the number and size of preadipocyte colonies as well as the total amount of triglycerides per well increased significantly in the GH group compared with the HX group. Because
the mature adipocyte counts in all three groups decreased with time, the observed increase in triglyceride content of culture wells could only be due to more preadipocyte colony formation under the influence of IBMX-Dex. Preadipocytes are the immediate precursors of adipocytes, and in preadipocyte cell lines the transition to mature adipocytes takes ~6–10 days. The cells express various transcription factors and then undergo postconfluent mitosis, clonal expansion, and growth arrest, but no overt adipocyte conversion occurs during the induction phase (15, 16). However, Smas et al. (26) showed that Dex induces downregulation of pref-1 mRNA during the induction phase, which is necessary before the appearance of the adipogenic transcription factors. Although no increase in adipocyte counts was noted, the expression of mRNA for PPARγ2, adipin, and leptin was increased in GH rats 2 days after removal of IBMX-Dex and persisted. Thus it is expected that there will be a lag period between the decrease in adipocyte number in culture and the appearance of new, differentiated, mature adipocytes. This will account for our observation that there was no increase in mature adipocyte numbers in the GH group even after stimulation with IBMX-Dex. The increase in preadipocyte colony counts and size after induction with IBMX-Dex, on the other hand, reflects the stimulation of uncommitted stromal cells into the adipocyte lineage. These cells then have to go through the preadipocyte stage before proceeding to mature, terminally differentiated adipocytes. In the HX rats, the absence of growth hormone seems to promote as well as accelerate the differentiation of the uncommitted pool of stromal cells into adipocytes. At confluence, most of the stromal cells derived from HX rats have been converted into adipocytes due to signals received in vivo, leaving few cells available for induction of adipogenesis in vitro after stimulation with IBMX-Dex. With growth hormone treatment of hypophysectomized rats, however, this process is abrogated. The uncommitted stromal cells are not depleted or primed to undergo accelerated adipogenesis and respond strongly to the induction agents. Thus, as expected and in line with our hypothesis, there was significantly more in vitro induction observed in the GH group than in the HX group, as evidenced by the increased preadipocyte colony formation and adipocytic markers.

Both adipocytes and preadipocytes can undergo dedifferentiation under appropriate stimulation conditions (2). In humans and rats, osteopenia induced by growth hormone deficiency is improved by growth hormone administration (5, 14). This could occur by one of two mechanisms: stimulation of uncommitted stromal cells to differentiate into osteoblasts or dedifferentiation of preadipocytes and some adipocytes into osteoblasts. Adipocytes that have not reached terminal differentiation are capable of dedifferentiation (15). The data presented here suggest that there was a pool of committed stromal cells that were still undergoing differentiation together with preadipocytes. This would account for the significantly higher number of adipocytes at confluence in the HX group compared with either the GH or CT groups. Thus it would appear that there is a population of committed marrow cells and preadipocytes under conditions of growth hormone deficiency that could be induced to dedifferentiate into osteogenic lineage under the influence of growth hormone administration.

In this study, we have used hypophysectomized rats as a model of growth hormone deficiency to investigate adipogenesis in the absence or presence of growth hormone. There are two possible confounding factors that could have accounted for our findings. First, hypophysectomy leads to deficiency of other pituitary hormones in addition to growth hormone, such as adrenocorticotropic hormone (ACTH) and prolactin, which could have contributed to the observed alterations in bone marrow adipogenesis. However, this is unlikely, because the lack of ACTH and prolactin would have affected both groups of hypophysectomized rats equally. Moreover, the only experimental variable between the two groups of hypophysectomized rats was the administration of growth hormone to one and not the other. Second, hydrocortisone is an adipogenic agent, and its administration could theoretically have affected our data. The fact that both groups of hypophysectomized rats received the same dose of hydrocortisone in replacement for the lack of cortisol due to the absence of ACTH means that there will be no preferential effects in one group compared with the other.

In conclusion, growth hormone administration to hypophysectomized rats resulted in inhibition of bone marrow adipogenesis in vivo, which persisted when bone marrow stromal cells were grown to confluence in vitro. However, although significantly more preadipocyte colonies were formed in the GH group when postconfluent cells were stimulated with IBMX-Dex, the in vivo signals received from growth hormone administration seemed to block their progression to fully matured adipocytes. Further studies will be needed to characterize the nature and mechanism of this differentiation arrest. During the review of this article, a study using growth hormone-deficient (dwo/dw) rats made similar observations concerning the effect of growth hormone on marrow adipocyte development (7a), consistent with the present report.

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