IGF-binding protein-5 stimulates osteoblast activity and bone accretion in ovariectomized mice

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Received 7 December 2000; accepted in final form 16 March 2001

Andress, Dennis L. IGF-binding protein-5 stimulates osteoblast activity and bone accretion in ovariectomized mice. Am J Physiol Endocrinol Metab 281: E283–E288, 2001.—Insulin-like growth factor binding protein-5 (IGFBP-5) is an osteoblast secretory protein that becomes incorporated into the mineralized bone matrix. In osteoblast cultures, IGFBP-5 stimulates cell proliferation by an IGF-independent mechanism. To evaluate whether IGFBP-5 can stimulate osteoblast activity and enhance bone accretion in a mouse model of osteoblast insufficiency, daily subcutaneous injections of either intact [IGFBP-5 (intact)] or carboxy-truncated IGFBP-5 [IGFBP-5-(1–169)] were given to ovariectomized (OVX) mice for 8 wk. Femur and spine bone mineral density (BMD), measured every 2 wk, showed early and sustained increases in response to IGFBP-5. Bone histomorphometry of cancellous bone showed significant elevations in the bone formation rate in both the femur metaphysis [IGFBP-5-(1–169)] and spine compared with OVX controls. IGFBP-5 also stimulated osteoblast number in the femur IGFBP-5-(1–169) and spine. These data indicate that IGFBP-5 effectively enhances bone formation and bone accretion in OVX mice by stimulating osteoblast activity. The finding that IGFBP-5-(1–169) is bioactive in vivo indicates that the carboxy-terminal portion is not required for this bone anabolic effect.

bone formation; bone mineral density; estrogen deficiency

INSULIN-LIKE GROWTH FACTOR (IGF) binding protein-5 (IGFBP-5), which is secreted by osteoblasts (3) and accumulates in mineralized bone matrix (8), may be important in normal bone remodeling on the basis of in vitro (4, 17) and recent in vivo (19) studies. Initial reports identified a 23-kDa, carboxy-truncated fragment of IGFBP-5 that was capable of stimulating osteoblast mitogenesis by an IGF-independent mechanism (4) and a similar sized recombinant form, [IGFBP-5-(1–169)], that was also able to stimulate osteoblast mitogenesis in the absence of IGF-I (6). More recent studies have shown that intact as well as truncated forms of IGFBP-5 (17, 23, 24) can variably stimulate osteoblast proliferation in the presence or absence of IGF-I. Although the action of IGFBP-5 on osteoblast activity has been attributed to both IGF-dependent and -independent mechanisms, it has been proposed that a membrane protein specific for IGFBP-5 binding (1) may mediate its IGF-independent effects through a unique signaling pathway (2).

Consistent with the in vitro findings are the recent results in normal mice showing that injections of recombinant human intact IGFBP-5 cause elevations in circulating osteocalcin and alkaline phosphatase (19). Because serum IGF-I levels did not change during the IGFBP-5 treatments, the data suggest that IGFBP-5 may stimulate bone formation directly. From this study, it was not possible, however, to determine whether the intact form of IGFBP-5 or one of its proteolytic fragments was responsible for the apparent anabolic effect, because much of the injected IGFBP-5 appeared in serum as fragmented components (19).

To further evaluate the in vivo effects of IGFBP-5 on bone remodeling, two forms of human recombinant IGFBP-5, IGFBP-5 (intact) and IGFBP-5-(1–169), were injected into ovariectomized (OVX) mice to determine whether the depressed bone formation of estrogen deficiency (11) can be stimulated by IGFBP-5. We found that the reduced bone formation rate in OVX mice was stimulated by IGFBP-5 treatment as a result of its stimulatory effect on osteoblast activity.

MATERIALS AND METHODS

Animals and experimental design. C3H mice (2 mo old) were obtained from Simonsen Laboratories (Gilroy, CA) 1 wk after OVX or sham OVX surgery. Throughout the study, all mice received standard rodent chow (5001, Purina, St. Louis, MO) and water ad libitum and were maintained in a 12:12-h light-dark cycle in an American Association for Accreditation of Laboratory Animal Care-accredited facility. After a 1-mo stabilization period, the mice were randomized into four treatment groups: 1) sham + phosphate buffered saline (PBS; n = 12), 2) OVX + PBS (n = 20), 3) OVX + IGFBP-5 (intact) (n = 10), and 4) OVX + IGFBP-5-(1–169) (n = 10). Human recombinant IGFBP-5 (intact) and IGFBP-5-(1–169) (Chiron, Emeryville, CA) were prepared as previously described (5), dissolved in sterile PBS, and stored in aliquots in siliconized freezer tubes (Genemate, ISC Bioexpress, Kaysville, UT) at −20°C. IGFBP-5 was given as daily subcutaneous injections at a dose of 30 μg/mouse each day for 8 wk; sham and OVX controls received similar injection volumes of...
PBS (200 μl). Bone mineral density (BMD; PixiMus, Lunar, Madison, WI) of the lumbar spine (L2–4) and both femurs was obtained on days 0, 14, 28, 42, and 56 (day of euthanasia). Precision at both sites is ±2%, according to the manufacturer’s specifications. The reported femur BMD results include the mean of both femurs for each animal. On days 48 and 53, calcein (30 mg/kg) and demeclocycline (25 mg/kg), respectively, were given intraperitoneally to later determine bone formation in the tissue sections. Animals were killed by cervical dislocation, and bones were removed for histomorphometric analysis. The study was approved by the Animal Subcommittees at the Veterans Affairs Puget Sound Health Care System and the University of Washington.

Bone histomorphometry. Lumbar spine (L2–4) and femoral bones were removed immediately after the animals were killed and were fixed, dehydrated, and embedded in methyl methacrylate according to previously described methods (5). Sections of the undecalcified bone were made using a heavy-duty microtome with a tungsten carbide knife. Ten-micrometer sections were used for fluorescent microscopy of the fluorochrome labels, and 7-μm sections were used for Goldner’s staining for evaluation of the static parameters and for tartrate-resistant acid phosphatase staining of osteoclasts.

Dynamic measurements were taken from the cancellous portions of the spine (L2–3) and from the distal femur metaphysis by use of a computerized digitizer and customized software (Bioquant; Biometrics, Nashville, TN). All measurements (dynamic and static) were performed under conditions in which the observer was blinded to the experimental groups by random slide labeling. The dynamic parameters included mineral apposition rate (MAR), or distance between the fluorochrome labels divided by the time interval between the two labels, and double fluorochrome label length, expressed as length per bone area. The bone formation rate (BFR) was calculated by multiplying the MAR by the double fluorochrome label length.

Static histomorphological parameters that were measured included mineralized bone area (as percentage of tissue area), osteoid area (as percentage of total bone area), osteoid width (in micrometers), and osteoblast surface (as length per total bone surface). Osteoclasts were quantified as the number per total bone surface as identified by positive (red or pink) staining of cells immediately adjacent to the bone surface.
Statistics. Results are expressed as means ± SE. Differences between groups were determined by ANOVA for normally distributed data or by a Kruskal-Wallis test for non-parametric data, with \( P < 0.05 \) taken as the minimum level of significance. Percent increases in BMD were calculated by subtracting the baseline value from later values and then dividing by the baseline value. This percent increase in BMD was then divided by the number of weeks since the baseline value.

RESULTS

Ovariectomy resulted in a significant decrease in bone accretion in both the femur and lumbar spine (Fig. 1). Femur BMD in the OVX + PBS group increased by a rate that was \( \sim 50\% \) less than in the sham + PBS group (1.3% vs. 3%/wk, \( P < 0.01 \)) and spine BMD increased by a rate that was \( \sim 35\% \) less than in the sham + PBS group (1.3% vs. 2.1%/wk, \( P < 0.05 \)), indicating that estrogen deficiency decreases bone accretion in growing mice.

In both IGFBP-5 treatment groups (Fig. 2) femur BMD rose by similar amounts by the end of 8 wk, and it was significantly greater than the OVX + PBS group (2.3% vs. 1.3%/wk, \( P < 0.05 \)). Because the BMD changes appeared to be different during the latter half of the study, further analysis of the change in BMD at 4 and 8 wk was performed. As seen in Fig. 3, femur BMD was completely preserved by IGFBP-5 treatment during the first 4 wk (Fig. 3A) but less so during the subsequent 4 wk (Fig. 3B). Similar findings were apparent for the spine BMD responses to IGFBP-5 treatment (Fig. 3, C and D). Overall, the preservation of the change in BMD as a result of IGFBP-5 treatment appeared to be more favorable in the femur than in the spine. The total skeletal BMD (excluding the skull) was not different among the four groups at the end of the study [range: from 0.068 ± 0.002 to 0.070 ± 0.001 gm/cm², \( P = \) not significant (NS)], indicating that this approximation of total body calcium was not affected. Two mice in the IGFBP-5-(1–169) treatment group died at the 6-wk time point during the anesthesia for BMD.

The bone histomorphometric changes were in agreement with the BMD findings with a few exceptions (Fig. 4). The OVX + PBS group had a mean BFR that was \( \sim 33\% \) lower than the sham + PBS controls (\( P < 0.05 \)), indicating that estrogen deficiency causes decreased bone remodeling in growing mice. Treatment with IGFBP-5-(1–169), but not with IGFBP-5 (intact), resulted in a higher BFR in the femur compared with OVX + PBS controls (\( P < 0.05 \)). Spine BFR, however, was well preserved by both IGFBP-5 treatments after 8 wk.

As shown in Table 1, the MAR in the femur metaphysis was not affected by ovariectomy or by IGFBP-5 treatment. The same finding was present in the spine (data not shown). Thus the changes in BFR induced by IGFBP-5 treatment noted in Fig. 4 were due to the increase in the amount of double fluorochrome labels present (\( P < 0.05 \)). This implies that IGFBP-5 treatment stimulates bone formation by increasing the number of osteoblasts that are actively forming new bone and not by increasing the activity of each osteoblast. Consistent with this notion is our finding that
the osteoblast surface was increased by IGFBP-5-(1–169) in the femur (Table 1, \( P < 0.05 \)) and by both forms of IGFBP-5 in the spine (Fig. 5).

The other, static histological parameters show that osteoid surface, osteoid width, and osteoclast number were not different among the groups at the end of the treatment period (Table 1). Notably, the small amount of osteoid that was identified is consistent with a rapid mineralization rate. Total bone area in the femur, which was reduced by ovariectomy, was preserved by IGFBP-5-(1–169) treatment (\( P < 0.05 \)). There were no significant differences in femur lengths or weight gain over the 8-wk treatment period among the four groups (range of weight gained: from 4.0 ± 0.9 to 4.8 ± 1.3 g, \( P = NS \)).

DISCUSSION

This study shows that IGFBP-5 stimulates bone accretion in OVX mice by a mechanism that involves the stimulation of bone surface osteoblasts and bone formation. These data are consistent with previous in vitro results demonstrating that IGFBP-5 stimulates

![Fig. 4. Bone formation rate in the femur (A) and spine (B) in response to treatment with IGFBP-5 (intact) and IGFBP-5-(1–169). *\( P < 0.05 \) vs. OVX/PBS.](image)

![Fig. 5. Osteoblast surface in the femur (A) and spine (B) in response to treatment with IGFBP-5 (intact) and IGFBP-5-(1–169). *\( P < 0.05 \) vs. OVX/PBS.](image)

Table 1. Histological parameters of cancellous bone in femur metaphysis of sham and OVX mice after 8 wk of treatment

<table>
<thead>
<tr>
<th></th>
<th>Sham PBS (n = 12)</th>
<th>OVX + PBS (n = 20)</th>
<th>OVX + Intact (n = 10)</th>
<th>OVX + 1–169 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone area, % tissue area</td>
<td>15 ± 1</td>
<td>11 ± 1*</td>
<td>13 ± 1</td>
<td>14 ± 1†</td>
</tr>
<tr>
<td>Osteoid surface, % of bone surface</td>
<td>3.1 ± 1.3</td>
<td>2.3 ± 1.0</td>
<td>1.0 ± 1.4</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td>Osteoid width, μm</td>
<td>5 ± 1</td>
<td>4 ± 1</td>
<td>3 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Osteoblast surface, % of bone surface</td>
<td>13.0 ± 2.4</td>
<td>7.1 ± 1.2*</td>
<td>9.0 ± 2.4</td>
<td>11.4 ± 1.2†</td>
</tr>
<tr>
<td>Osteoclast surface, no./mm bone surface</td>
<td>7.3 ± 1.3</td>
<td>7.0 ± 1.0</td>
<td>7.4 ± 1.3</td>
<td>8.3 ± 2.2</td>
</tr>
<tr>
<td>Mineral apposition rate, μm/day</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Double label length, μm/mm² bone area</td>
<td>1,236 ± 80</td>
<td>895 ± 56*</td>
<td>959 ± 118</td>
<td>1,161 ± 72†</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \) = no. of mice per group. Sham, sham ovariectomy (OVX); PBS, phosphate-buffered saline; Intact, intact insulin-like growth factor binding protein-5 [IGFBP-5 (intact)]; 1–169, carboxy-truncated IGFBP-5 [IGFBP-5-(1–169)]. These 4 groups are described in detail in MATERIALS AND METHODS. *\( P < 0.05 \) vs. sham + PBS; †\( P < 0.05 \) vs. OVX + PBS.
osteoblast proliferation (3, 4, 17) and extend recent studies showing the bone-stimulatory effects of IGFBP-5 in normal mice (19). The finding that IGFBP-5(1–169) was stimulatory in this model indicates that the presence of the carboxy-terminal portion of the molecule is not required for osteoblast activation. This is consistent with the recent finding that IGFBP-5(1–169) can bind to and stimulate the phosphorylation of a specific osteoblast membrane receptor (2) and suggests that intact IGFBP-5 may require a proteolytic step before it can stimulate osteoblast activity in vivo. Significant serum proteolysis of intact IGFBP-5 in mice has been shown to occur within 1 h of its injection (19).

Even though IGFBP-5 treatment was anabolic for bone without the addition of IGF-I, our study was not designed to exclude the possibility that IGF-I (circulating or osteoblast derived) may have contributed to IGFBP-5-stimulated bone formation. This would be possible if IGFBP-5 sequestered IGF-I to the bone surface to increase IGF receptor occupancy. However, several findings from other studies suggest that this is not the likely mechanism for the IGFBP-5 effect. For example, Mohan et al. (17) have shown that IGFBP-5 binding to the osteoblast surface does not increase the binding of 125I-labeled IGF-I to its receptor. Moreover, Zhao et al. (27) have shown that the increased bone formation rate in transgenic mice that overexpress osteoblast IGF-I results from the stimulation of the mineral apposition rate and not from the osteoblast number or the extent of double fluorochrome labels. This is in contrast to our results, in which IGFBP-5 was found to enhance bone formation by stimulating osteoblast number and, consequently, the extent of double labels (Table 1) without affecting the mineral apposition rate. Taken together, these data suggest that, in mouse bone, IGF-I may function as an osteoblast differentiation factor (stimulating collagen production and mineralization rate of each osteoblast), whereas IGFBP-5 may function more to enhance the number of osteoblasts that actively form mineral. Whether the IGFBP-5 effect derives from the stimulation of marrow preosteoblasts or from the activation of bone lining cells cannot be determined. Alternatively, IGFBP-5 may protect against osteoblast/osteocyte apoptosis.

In this model of estrogen deficiency, IGFBP-5(1–169) treatment appeared to stimulate bone accretion more in the femur than in the spine, although in both compartments there was less than full preservation of bone mineral density for the entire 8 wk of treatment (Fig. 3). This apparent diminished effect may have resulted from the development of neutralizing antibodies to human IGFBP-5 and/or preferentially increased bone resorption in the cortical bone compartment. The finding that IGFBP-5 treatment did not result in an increased in the mature osteoclast population supports the notion that cells of the osteoblast lineage are its principal target in bone. It is not possible to decide from this study whether femoral osteoblasts are more or less responsive to IGFBP-5 compared with those in the spine. However, the higher intrinsic bone formation rate in the femoral metaphysis suggests possible dynamic differences in osteoblast physiology that might result in a differential response to IGFBP-5.

This study adds to a growing body of work indicating that estrogen is important for maintaining normal osteoblast and osteocyte activity (10, 11, 25, 26). Although it is well known that estrogen deficiency causes increased bone resorption (7, 11) and that estrogen induces osteoclast apoptosis in vitro (15), recent studies have shown that estrogen withdrawal in humans causes osteocyte apoptosis (25) and that estrogen treatment in rodents stimulates bone formation (7, 9, 21, 22). Despite concerns that the growing murine skeleton may not completely mimic human bone remodeling, it has proved to be a useful model for studying the effects of estrogen on specific parameters of bone formation. Proposed mechanisms for this bone anabolic effect include estrogen-stimulated osteoblast proliferation and collagen synthesis (13), upregulation of osteoblast-secreted IGF-I (12, 14), and mediation of the effects of mechanical loading to increase bone formation (20), possibly acting through the osteocyte (18). Although it is unknown whether estrogen stimulates osteoblast-secreted IGFBP-5 directly, it may enhance IGFBP-5 production indirectly by increasing ambient osteoblast-derived IGF-I (12, which is known to stimulate IGFBP-5 mRNA expression in osteoblasts (16). Despite the uncertainty over the role of estrogen deficiency in causing reductions in bone formation, it is clear from this study that IGFBP-5 treatment can stimulate osteoblast activity in estrogen-deficient mice.

In summary, this study shows that the in vivo response of IGFBP-5 mimics its in vitro stimulatory effect in osteoblasts to cause enhanced bone formation in a mouse model of osteoblast insufficiency. Because its mechanism of stimulating bone formation is distinct from that ascribed to IGF-I in transgenic mice, these data indirectly support an IGF-I-independent mechanism of osteoblast stimulation by IGFBP-5.

The technical assistance of Qiao Sun, Carl Baker, and Dawn Moran is gratefully acknowledged. This work was supported by funds from the National Institutes of Health (RO1 AR-44911).

REFERENCES


