Insulin-Like Growth Factor-Binding Protein-1 Expression in Cultured Human Bone Cells: Regulation by Insulin and Glucocorticoid*

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ABSTRACT

Insulin-like growth factors (IGFs) and their specific regulatory binding proteins (IGFBPs) are postulated to play a key role in bone metabolism. To date, IGFBP-2 through -6 have been characterized in bone cell systems. In this study we focused on IGFBP-1.

Primary cultures of normal human osteoblasts derived from trabecular bone (hOB cells) expressed low levels of IGFBP-1 messenger RNA (mRNA), as determined by Northern analyses. Treatment of hOB cells with 1 μM cortisol or 100 nM dexamethasone for 20 h stimulated IGFBP-1 mRNA expression 5-fold and increased levels of immunoassayable IGFBP-1 in the conditioned medium 3-fold. Estradiol and progesterone had no effect. IGFBP-1 expression was not observed in U-2, TE-85, or MG-63 human osteosarcoma cell lines or in normal human fibroblasts. Insulin (1-100 nM) potently inhibited both basal and glucocorticoid-stimulated IGFBP-1 expression in hOB cells. Insulin had little or no effect on steady state levels of the other IGFBP mRNA. A monoclonal antibody to the insulin receptor blocked insulin binding to insulin receptors and completely prevented insulin-induced suppression of IGFBP-1.

In summary, we have documented IGFBP-1 mRNA and protein expression in normal nontransformed human osteoblastic cells. This expression was stimulated by glucocorticoids and inhibited by insulin in a manner similar to IGFBP-1 regulation in hepatocytes. Insulin acts through insulin receptors on hOB cells. We postulate that IGFBP-1 produced by osteoblasts in vivo can modulate local actions of IGF on bone formation in response to changes in glucocorticoid and insulin concentrations. (Endocrinology 137: 3295-3301, 1996)

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Materials and Methods

Materials

Crystalline human insulin was supplied by Eli Lilly Co. (Indianapolis, IN), and recombinant human (rh) IGF-I and IGF-II were purchased from R&D Systems (Minneapolis, MN) and Bachem (Torrance, CA), respectively. The following IGF analogs were kindly provided as follows: [Gln3,Ala4,Tyr5]IGF-I ([QAYL]IGF-I), Dr. M. Cascieri, Merck, Sharp, and Dohme (Rahway, NJ); [Leu27]IGF-II, Dr. S. Ling, The Whittier Institute (La Jolla, CA). [Long R3]IGF-I was obtained from GroPep (Adelaide, Australia). Monoclonal antibody to the type I IGF receptor (aIR-3) was obtained from Oncogene Sciences (Manhasset, NY), and monoclonal antibody to the insulin receptor (a7-9) was kindly provided by Dr. K. Siddle (University of Cambridge, Cambridge, UK). 3H[2]iodotyrosyl]14 insulin was obtained from Amersham Life Sciences (Arlington Heights, IL). The complementary DNA (cDNA)
IGFBP-1 assay

IGFBP-1 protein concentrations in conditioned media were measured using a two-site immunoradiometric assay kit (Diagnostic Systems Laboratories, Webster, TX). Inter- and intraassay CVs were 6.7% and 4.0%, respectively, at low IGFBP-1 concentrations, with a sensitivity of 0.11 ng/ml and no detectable cross-reactivity with hIGFBP-2, -3, -4, -5, and -6 or bovine serum.

Binding assays

Binding assays were performed directly on confluent hOB cell monolayers, as detailed previously (28, 29). Cells were washed three times with cold HEPES binding buffer, pH 7.4, and 0.5% BSA and then incubated with \[^{125}I\]IGF-I (25,000 cpm) without and with 100 ng/ml unlabeled [Leu\(^{6}\)]IGF-II at 15 C for 2.5 h. [Leu\(^{6}\)]IGF-II competes with \[^{125}I\]IGF-I for IGFBP binding and allows for estimates of specific IGF-I binding to receptor (30, 31). \[^{125}I\]Insulin binding was performed similarly, but at pH 8.0. Nonspecific binding was defined as the amounts of \[^{125}I\]IGF-I and \[^{125}I\]insulin bound in the presence of excess IGF-I (250 ng/ml) and insulin (400 ng/ml), respectively. Nonspecific binding was less than 1% of the total counts added and was subtracted from total binding to determine specific binding.

Affinity cross-linking

Confluent cultures (six-multilwell dishes) were washed three times with cold HEPES binding buffer and 0.5% BSA and then incubated with \[^{125}I\]insulin (100 cpm/ml), \[^{125}I\]IGF-I (1.25 \times 10^6 cpm/ml) or \[^{125}I\]Long R IGF-I (5 \times 10^5 cpm/ml) without and with unlabeled insulin, [Leu\(^{6}\)]IGF-II at 15 C for 2.5 h. Affinity cross-linking of monolayers with disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL) was performed as described previously (28). Reduced (plus 100 mM dithiothreitol) samples were electrophoresed using SDS-PAGE. Unstained M, standards (BioRad Laboratories, Richmond, CA) were processed in parallel. Gels were stained with Coomassie blue, dried, and exposed to film. Scanning densitometry and molecular size determinations were performed using an UltraScan XL laser densitometer and Gel-Scan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Statistical analysis

Data are presented as the mean ± se. ANOVA and Dunnett’s test were used when comparing multiple groups with a single control. Results were considered statistically significant at P < 0.05.

Results

IGFBP-1 expression in human bone cells

Figure 1 presents a Northern blot of total RNA extracted from normal hOB cells and hybridized with \[^{32P}\]labeled hu-
man IGFBP-1 cDNA. Cultured hOB cells had been incubated for 20 h in serum-free medium with and without 100 nM insulin, 100 nM dexamethasone, or their combination. A 1.6-kilobase (kb) IGFBP-1 transcript was clearly detectable in untreated (control) hOB cells. Insulin treatment resulted in a 50% decrease in steady state levels of IGFBP-1 mRNA (Table 1). On the other hand, dexamethasone treatment increased IGFBP-1 mRNA levels 5-fold. In the presence of insulin, however, the glucocorticoid-induced increase in IGFBP-1 was completely blocked, and no IGFBP-1 mRNA was detectable even with prolonged autoradiographic exposure. IGFBP-3 (2.6 kb), IGFBP-4 (2.2 kb), and IGFBP-5 (6.0 kb) mRNA levels were decreased by 50%, 35%, and 20%, respectively, with dexamethasone treatment of hOB cells as previously reported (9), although the inhibitory effect on IGFBP-5 mRNA was less evident, perhaps due to the different incubation times. The combination of insulin with dexamethasone did not influence these decreases in expression, whereas insulin alone only had a modest effect on IGFBP-3 mRNA (32% increase). Steady state levels of IGFBP-6 (1.3 kb) mRNA were not regulated by insulin or glucocorticoids in hOB cells during this incubation period, and the weak expression of IGFBP-2 mRNA is not shown. Specific glucocorticoid receptor-mediated action was indicated because dexamethasone was more potent than cortisol in stimulating IGFBP-1 expression, whereas β-estradiol and progesterone were without effect in hOB cells (Fig. 2).

Changes in IGFBP-1 gene expression in hOB cells were reflected in translated protein (Table 1). Dexamethasone treatment for 20 h increased immunoassayable IGFBP-1 levels in the conditioned medium 3-fold. Insulin treatment produced 55% lower levels, and insulin plus dexamethasone resulted in an 80% decrease to nearly undetectable levels of IGFBP-1. In eight experiments, conditioned medium from 40-h dexamethasone-treated hOB cells had 3.1 ± 0.7 rig/ml IGFBP-1, whereas medium from cells treated with dexamethasone in the presence of insulin had 0.8 ± 0.2 rig/ml. Low medium IGFBP-1 levels were associated with a significant increase in cell number (0.54 ± 0.02 · 10^5 for dexamethasone treatment vs. 0.66 ± 0.05 · 10^5 for dexamethasone in the presence of insulin). Immunoblot studies using an IGFBP-1-specific monoclonal antibody confirmed the presence of an ~30-kDa band in hOB-conditioned medium after dexamethasone treatment (data not shown).

To determine the distinctiveness of these findings for normal human osteoblastic cells, normal human fibroblasts as well as transformed hOB cells and human osteosarcoma cells were similarly treated with and without 100 nM dexamethasone for 20 h. No IGFBP-1 message was detectable in fibroblasts or in MG-63, TE-85, U2, human osteosarcoma cells; HOBIT, SV40-transformed hOB cells; FOB, SV40 transformed fetal human osteoblast-like cells.

Table 1. Regulation of IGFBP-1 in hOB cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>mRNA^a</th>
<th>Protein^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>0.7</td>
</tr>
<tr>
<td>Ins</td>
<td>50</td>
<td>0.3</td>
</tr>
<tr>
<td>Dex</td>
<td>500</td>
<td>1.9</td>
</tr>
<tr>
<td>Dex + Ins</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Ins, Insulin (100 nM); Dex, dexamethasone (100 nM); ND, not detectable.
^a Percentage of control (densitometry scan of Northern blot in Fig. 1).
^b Nanograms per ml (IGFBP-1 IRMA of 20-h hOB cell-conditioned medium from the experiment in Fig. 1).

Table 2. IGFBP mRNA expression in human bone cells and fibroblasts

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>BP-1</th>
<th>BP-3</th>
<th>BP-4</th>
<th>BP-5</th>
<th>BP-6</th>
</tr>
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<tbody>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HF</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MG-63</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TE-85</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>U-2</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HOBIT</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>FOB</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

hOB, Normal adult human osteoblast-like cells; HF, normal adult human fibroblasts; MG-63, TE-85, U2, human osteosarcoma cells; HOBIT, SV40-transformed hOB cells; FOB, SV40-transfected fetal human osteoblast-like cells.
^a Shown is the relative expression determined by Northern analysis of RNA after 20-h serum-free incubation with and without 100 nM dexamethasone. +, Strong; +/−, weak; −, undetectable; ND, not determined.
Receptor-mediated suppression of IGFBP-1 expression

As shown in Fig. 3, 10 nM each of insulin, IGF-I, and IGF-II had similar inhibitory effects on basal and dexamethasone-induce IGFBP-1 mRNA expression. These results prompted investigation into which receptor mediates IGFBP-1 gene suppression in hOB cells. Cultured hOB cells bound radiolabeled insulin and IGF-I. Total specific [125I]insulin binding (n = 4) was 0.29 ± 0.013%/10^5 cells. Total specific [125I]IGF-I binding (n = 4) was 6.3 ± 0.34%/10^5 cells, but in the presence of unlabeled [Leu²⁷]IGF-II, specific binding was only 1.1 ± 0.06%/10^5 cells. [Leu²⁷]IGF-II competes for [125I]IGF-I binding to IGFBPs, but not type I IGF receptors (30, 31). Thus, greater than 80% of [125I]IGF-I binding appeared to be to IGFBPs on the hOB cell surface. Competitive binding studies were verified by affinity cross-linking (Fig. 4). Under reducing conditions, [125I]insulin bound predominantly to a 135-kDa protein, the labeling of which was displaceable by unlabeled insulin but not by unlabeled IGF-I, indicating insulin binding to the ~135-kDa α-subunit of the insulin receptor on these cells (32). [125I]IGF-I also bound to a 135-kDa protein as well as to proteins at 40–50 kDa. Labeling of the 135-kDa band was displaceable by IGF-I and insulin. Labeling of the lower molecular sized bands was displaceable by IGF-I, but not by insulin. Unlabeled [Leu²⁷]IGF-II (100 ng/ml) effectively inhibited [125I]IGF-I binding to 40–50-kDa bands by 76%, but had no effect on binding to the 135-kDa band. These results indicate that IGF-I binds to the 135-kDa α-subunit of the type I IGF receptor on hOB cells and to 40–50-kDa cell-associated IGFBPs (33).

Indeed, cell-associated IGFBP was the predominant [125I]IGF-I-binding form. IGF-I receptor binding was verified by cross-linking of [125I]-Long R²]IGF-I to hOB cells. This IGF-I analog binds to type I IGF receptor, but poorly to IGFBPs (34), and was mainly cross-linked to the 135-kDa band. Thus, cultured hOB cells possess specific insulin and type I IGF receptors as well as cell-associated IGFBP.

To distinguish between the two receptor types, we used antibodies relatively specific for the human type I IGF receptor (aIR-3) (35) and the human insulin receptor (α47-9) (36). As shown in Table 3, α47-9 completely blocked [125I]insulin receptor binding while only inhibiting 25–30% of [125I]IGF-I receptor binding (i.e. binding in the presence of [Leu²⁷]IGF-II). aIR-3 inhibited [125I]IGF-I binding to receptor by ~50% without affecting [125I]insulin binding. hOB cells were pretreated for 1 h with α47-9 or aIR-3 before the addition of insulin, [QAYL]IGF-I, or [QAYL-Leu²⁷]IGF-II, each at 1-nM final concentrations. The IGF-I and IGF-II analogs were used to bypass the effects of soluble or cell-associated IGFBP on specific IGF-I and IGF-II receptor interactions (37, 38). Dexamethasone (100 nM) was present under all conditions. As shown in Table 4, 1 nM insulin and 1 nM [QAYL]IGF-I significantly inhibited dexamethasone-induced IGFBP-1 production by ~40%, whereas [QAYL-Leu²⁷]IGF-II had no effect. α47-9 completely prevented insulin-induced suppression of IGFBP-1 and reversed
TABLE 4. Receptor-mediated IGFBP-1 suppression

<table>
<thead>
<tr>
<th></th>
<th>IGFBP-1</th>
<th>a47-9</th>
<th>aIR-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>101 ± 0.3</td>
<td>94 ± 7.3</td>
</tr>
<tr>
<td>Dex</td>
<td>56 ± 2.5 °</td>
<td>97 ± 1.0</td>
<td>58 ± 3.0 °</td>
</tr>
<tr>
<td>Dex + Ins</td>
<td>60 ± 2.9 °</td>
<td>78 ± 0.6 °</td>
<td>66 ± 3.2 °</td>
</tr>
<tr>
<td>Dex + Q-IGF-I</td>
<td>98 ± 2.6</td>
<td>99 ± 6.9</td>
<td>100 ± 3.6</td>
</tr>
<tr>
<td>Dex + Q-IGF-II</td>
<td>98 ± 2.6</td>
<td>99 ± 6.9</td>
<td>100 ± 3.6</td>
</tr>
</tbody>
</table>

hOB cells were pretreated for 1 h with a47-9 (1:24 dilution) or aIR-3 (1 µg/ml) before the indicated treatment. The 26-h serum-free condition media were assayed for IGFBP-1 by IRMA. Dex, Dexamethasone (100 nM); Ins, insulin (1 nM); Q-IGF-I, [QAYL]IGF-I (1 nm), binds type I IGF receptor, does not bind IGFBPs (37); Q-IGF-II [QAYL-Leu22]IGF-II (1 nm), binds type II IGF receptors, does not bind type I IGF receptors or IGFBPs (38).

Results are the mean ± SEM of three experiments expressed relative to IGFBP-1 values in the Dex-treated control (1.3 ± 0.2 ng/ml) P < 0.05 vs. control.

Discussion

This study provides definitive evidence that normal hOB-like cells in culture express IGFBP-1 mRNA and protein. This conflicts with a number of articles indicating that IGFBP-1 is not expressed in bone cells. The disparate results are probably due to the different cell models and culture conditions. Highly heterogeneous cell populations may have undetectable signal, as IGFBP-1 is not expressed by human fibroblasts even under stimulated conditions (39). IGFBP-1 also is not expressed in the various transformed human osteoblastic cell models commonly used for such studies. Species specificity and developmental regulation of the IGFBP-1 response in bone might contribute to the differing results as well (2).

IGFBP-1 expression has been detected in only a few tissues. In humans, expression was initially detected in liver and the reproductive tract, and later in the kidney (18-21); our work adds bone to this short list. In hepatocytes, basal IGFBP-1 transcription appears to require hepatic nuclear factor 1 (HNF1) or related family members (40-42), and this protein family is possibly also important in basal IGFBP-1 transcription in endometrial stromal cells (43). HNF1 is believed to be a master regulatory protein required to produce and maintain the hepatic phenotype; significant expression of HNF1 family members in kidney suggests that HNF1 proteins may play a similar role in specialized kidney epithelial cells (41). Although it is possible that HNF1 proteins are expressed in hOB, recent studies suggest that the IGF-I promoter is quite active in hepatocytes in the absence of the HNF1 DNA-binding domain, and thus in the absence of a HNF1 effect, if high concentrations of glucocorticoids are present (Scheimann, A. O., and D. R. Powell, unpublished observations). In fact, IGFBP-1 expression in hOB becomes prominent only with high glucocorticoid concentrations (9). These observations suggest that IGFBP-1 expression may be detected in other tissues, even those not expressing HNF1 family members, if the tissues are exposed to high concentrations of glucocorticoids.

Regulation of IGFBP-1 expression in hOB cells appears similar to that in hepatocytes. In human and rat hepatocytes, glucocorticoids increase IGFBP-1 expression, whereas insulin inhibits both basal and glucocorticoid-stimulated IGFBP-1 expression; in each case, hormonal regulation appears to be at the level of transcription (16, 17). Likewise, glucocorticoids increased IGFBP-1 mRNA and protein levels in hOB cells, whereas insulin acted as a dominant negative regulator of IGFBP-1 expression. The effects of glucocorticoids and insulin on IGFBP-1 expression in hOB cells were unique to this IGFBP; dexamethasone decreased IGFBP-3, -4, and -5 mRNA levels, with no effect on IGFBP-6 levels, and in the presence of dexamethasone, the further addition of insulin had no effect on mRNA levels for any of these IGFBPs. Interestingly, insulin appeared to be a more effective inhibitor of IGFBP-1 expression in the presence of dexamethasone, although the basis for this is unclear. In human hepatocytes, the effects of glucocorticoid and insulin on IGFBP-1 promoter activity are conferred through two proximal glucocorticoid response elements that flank a single insulin response element (17). Although it is likely that the same elements are responsible for glucocorticoid and insulin regulation of IGFBP-1 expression in hOB cells, additional experiments are required to confirm this hypothesis.

To determine which receptor type mediates the inhibitory effect of insulin on IGFBP-1 expression in human bone, we first identified specific type I IGF and insulin receptors on hOB cells. Previous studies had examined total [125I]IGF-I specific binding, which would include binding to both type I IGF receptors and IGFBPs. Indeed, the competitive binding and affinity cross-linking studies presented here show that cell-associated IGFBP can account for greater than 80% of [125I]IGF-I binding to hOB cells. Parallel studies demonstrated specific insulin receptors on hOB cells. There are no published data for insulin binding to receptors in bone cells, although physiological concentrations of insulin and IGF-I have been shown to have equivalent effects on rat bone collagen synthesis (44). As shown by the antibody studies, insulin inhibition of IGFBP-1 is mediated through the insulin receptor. Specific insulin receptor-blocking monoclonal antibody (a47-9) completely prevented insulin-induced suppression of IGFBP-1 expression in hOB cells. The apparent equimolar inhibition of IGFBP-1 by IGF-I is not entirely explained, as a47-9 only partially reversed [QAYL]IGF-I-induced suppression of IGFBP-1, and the IGF-I receptor-blocking monoclonal antibody (aIR-3) had no effect. However, as aIR-3 was generated against the fetal form of the type I IGF receptor (45), and hOB cells in our studies are from adult bone, we cannot exclude the possibility of a type I IGF receptor-mediated action. Insulin does, but IGF-I does not, lower IGFBP-1 mRNA levels in primary rat hepatocytes (46), cells that express insulin receptors but not type I IGF receptors. However, IGF-I significantly lowers IGFBP-1 mRNA levels in HepG2, human hepatoma cells (47), which have abundant type I IGF receptors, and Poretsky et al. (48) recently reported that insulin receptors do not mediate the inhibitory effect of IGF-I on IGFBP-1 production in human granulosa cells. These data suggest that in cells such as hOB and granulosa cells, local IGF levels may regulate IGFBP-1 expression through the type I IGF receptor. [QAYL-Leu22]IGF-II, which binds specifically to the type II IGF re-
ticoids, must be considered as a potential major player in bone metabolism and could have a significant role in the associated with prolonged fasting, glucocorticoid excess, and reduced by osteoblasts and regulated by insulin and glucocorticoid stimulation and the potent inhibitory action of insulin, it may be that osteoblast IGFBP-1 production is minimal under normal physiological circumstances. However, in conditions of high integrated glucocorticoid exposure and/or insulin deficiency or resistance, hOB production of IGFBP-1 may be sufficient to influence the actions of IGF on bone. In this respect, it is interesting to postulate a role for osteoblast IGFBP-1 production in the pathogenesis of bone disease associated with prolonged fasting, glucocorticoid excess, and poorly controlled insulin-dependent diabetes mellitus. Indeed, elevated glucocorticoid levels have been identified as a major factor in reduced tibial growth and increased plasma IGFBP-1 in streptozotocin-induced diabetic rats, but not in insulin-replete rats (51).

In conclusion, our data clearly show that IGFBP-1, produced by osteoblasts and regulated by insulin and glucocorticoids, must be considered as a potential major player in bone metabolism and could have a significant role in the pathogenesis of bone disease.

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References

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