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INTACT BUT NOT TRUNCATED INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 (IGFBP-3) BLOCKS IGF I-INDUCED STIMULATION OF OSTEOBLASTS: CONTROL OF IGF SIGNALLING TO BONE CELLS BY IGFBP-3-SPECIFIC PROTEOLYSIS?

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IGFBP-3 is the predominant IGFBP in serum and the major IGFBP secreted by osteoblasts. Native and recombinant IGFBP-3 and a truncated form lacking the carboxyterminal third were tested for their effects on 2 osteoblastic cell lines. Intact but not truncated IGFBP-3 blocked IGF I-stimulated DNA and glycogen synthesis. Inhibition was dose-dependent and found whenever the concentration of intact IGFBP-3 exceeded the concentration of IGF I. Truncated IGFBP-3 appears to result from proteolytic cleavage and does occur in vivo. The loss of inhibition by IGFBP-3 may be regulated at the site of IGF target cells and thus be essential for IGF I-induced osteoblast growth.

Bone contains insulin-like growth factors (IGF I and IGF II) (1). IGFs contribute to growth of normal osteoblasts (2) and of osteogenic sarcoma cells (3). IGFs and specific IGF binding proteins (IGFBPs) are synthesized in bone. As IGFBPs may inhibit or enhance action, we looked for effects of purified recombinant and native IGFBP-3 on osteoblastic cells, using two cell lines (rat: PyMS, and human: Saos-2/B-10). Among all the IGFBPs, IGFBP-3 is the most abundant not only in sera (4) but also in media conditioned by these 2 particular cell lines.

IGFBPs have been isolated from adult rat serum by Sepharose-IGF I affinity chromatography and HPLC purification (5). In the rat, IGFBP-3 is represented by several molecular forms (6). Amino acid sequencing revealed that both the intact IGFBP-3 (42/45 kDa) as well as a 31 kDa IGFBP had NH_2-terminal amino acid sequences identical to those later predicted from molecular cloning of rat IGFBP-3 cDNA (7). It is likely that the 31 kDa IGFBP is truncated IGFBP-3, lacking the carboxyterminal third (5).

Among the IGFBPs purified from adult human serum by Sepharose-IGF I affinity column and HPLC (8), the most hydrophilic BP has an approximate

Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; BSA, bovine serum albumin.

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molecular mass of 31 kDa. NH_2-terminal sequences of the 31 kDa protein, the 42/45 kDa band and of IGFBP-3 (9) are identical. Like the 42/45, the 31 kDa protein is N-glycosylated.

During purification of IGFBP-3 from human serum, some loss of the characteristic 42/45 kDa forms (2 glycosylation variants) of IGFBP-3 occurs, and, at the same time, a relative increase in the 31 kDa form. The findings are consistent with degradation of IGFBP-3, apparently by specific cleavage. This results in a truncated form of IGFBP-3, which represents the amino terminal two thirds of the molecule.

IGFBP-3 and truncated IGFBP-3 were compared for their modulatory effects on the response of osteoblastic cells to IGF I.

MATERIALS AND METHODS

Test compounds:
Human insulin was a gift from Novo-Nordisk, Gentofte DK. Recombinant human (rh)IGF I was from Ciba-Geigy, Basel, rhIGFBP-3 was a gift from Dr. A. Sommer, Biogrowth, Richmond CA (10). Rat IGFBP-3 and rat and human COOH-truncated (31 kDa) IGFBP-3 had been prepared for amino acid sequence analysis; their isolation and characterization have been reported previously (5,8). During HPLC truncated hIGFBP-3 elutes in fractions 36-40 (8). 36-38 and 39+40 were pooled. The concentrations indicated in the figures are based on estimations of the amount of purified protein analysed by silverstaining and confirmed by amino acid analysis.

Cells:
PyMS cells, a rat bone-derived cell line (2), were obtained from Drs. A. Lichtler and D. Rowe, University of Connecticut, Farmington USA. They were grown in DMEM medium containing 2 mM glutamine and 5 % FCS.
Human osteoblastic osteosarcoma (Saos-2/B-10) cells were a gift from Drs. S. and G.A. Rodan (11,12). They were passaged in DMEM/Ham's F 12 medium (1:1 mixture) containing 2 mM glutamine and 10 % FCS.

^{3}H-thymidine incorporation into DNA:
PyMS and B-10 cells were plated at a density of 0.2 mio per 35 mm diameter dish (Falcon) in 5 % FCS DMEM and 10 % FCS DMEM/F 12, respectively. After 2 days, confluent monolayers had formed, and the medium was replaced with serum-free Ham's F 12 medium for 30 min prior to exposure of the cells to test medium, F 12 supplemented with charcoal-treated bovine serum albumin (BSA, at 1 g/l). After incubation for 18 h in test media containing IGF I and IGFBPs as indicated, the cells were pulsed with [^{3}H]thymidine (Amersham, 80 Ci/mmol; 1 µCi/dish), incubated for 3 h at 37°C, rinsed with cold PBS, and DNA was precipitated with 10 % TCA. Incorporated radioactivity was measured in a β-counter.

^{14}C-glucose incorporation into glycogen:
B-10 cells were plated at a density of 0.2 mio per 35 mm diameter dish (Falcon) in 10 % FCS DMEM/F 12. After 2 days, the medium was replaced with serum-free Ham's F 12 medium for 30 min prior to exposure of the cells to test medium, F 12 supplemented with 1 g/l BSA, test compounds and ^{14}C-glucose (1 µCi/dish). After 4 h, the cells were quickly rinsed with cold PBS and glycogen was precipitated as described elsewhere (13). Incorporated radioactivity was measured in a β-counter.

RESULTS AND DISCUSSION

PyMS cells are a cell line derived from rat bone. IGF I increases incorporation of ^{3}H-thymidine into DNA in dose-dependent manner, with a half-maximal stimulation at 0.5 nM (2). The first experiments were designed to test rat IGFBP-3 preparations at concentrations estimated to be equimolar to IGF I. No effects of rat IGFBP-3 itself were observed. However, it inhibited 1 nM IGF I-stimulated DNA synthesis in a dose-dependent manner. Half-maximal inhibition occurred at about 1 nM BP-3 both with the native rat protein and with rhIGFBP-3 (not shown). At 10 nM, inhibition was complete. Similar to intact rat IGFBP-3, truncated (31 kDa) rat IGFBP-3 alone had no significant effect, however, in contrast to intact IGFBP-3, it further enhanced the stimulatory effect of IGF I on DNA synthesis (Fig. 1). Thus, full-length IGFBP-3 and truncated IGFBP-3 differed completely in their biological activity: IGFBP-3 inhibited and truncated IGFBP-3 enhanced IGF-stimulated DNA synthesis.

The corresponding truncated (31 kDa) IGFBP-3 purified from human serum (8) was tested for its biological activity on a human osteosarcoma cell line (Saos-2/B-10) and compared with that of recombinant human IGFBP-3. Similar to PyMS cells, B-10 cells are sensitive to IGF I. Maximal stimulation of DNA synthesis is observed at 3 nM, half-maximal stimulation at 0.2 nM. Again, rhIGF I was tested at 1 nM, in the presence of 10 nM rhIGFBP-3 or its trun-

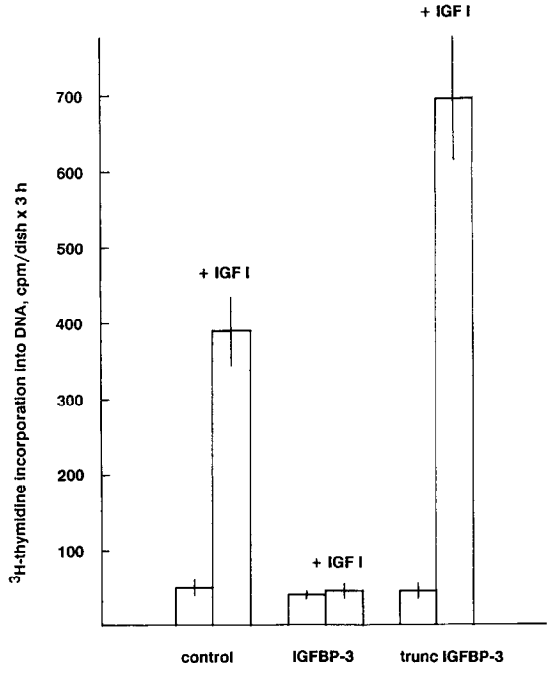

Fig. 1. Modulation of 1 nM rhIGF I-stimulated DNA synthesis in rat osteoblastic cells by full-length and truncated rat IGFBP-3 (10 nM). Columns indicate the mean of n = 9, bars the SEM, from 3 experiments in triplicate.

TABLE I

Stimulation of DNA synthesis in human osteoblastic (B 10) cells by 1 nM rhIGF I in the absence and presence of rhIGFBP-3 or truncated (tr) hIGFBP-3

	control	+ IGF I, 1 nM
a) control	10403 ± 1102	63492 ± 10875
rhIGFBP-3, 10 nM	10468 ± 1178	17246 ± 3218
tr BP-3 (fr 36-38)	23875 ± 2963	71647 ± 11355
b) control	9587 ± 580	45492 ± 1232
rhIGFBP-3, 10 nM	9154 ± 344	10296 ± 333
tr BP-3 (fr 39+40)	43696 ± 2783	47876 ± 2197
c) control	8005 ± 571	29497 ± 1396
tr BP-3, 0.1 nM	8783 ± 705	31523 ± 1361
tr BP-3, 0.3 nM	10632 ± 1194	34359 ± 1599
tr BP-3, 1 nM	14213 ± 1575	34600 ± 2616
tr BP-3, 3 nM	22355 ± 1580	39237 ± 2705
tr BP-3, 10 nM	30498 ± 1385	34970 ± 1647

Cells were exposed to test media for the last 21 h and pulsed with ^{3}H-thymidine for the last 3 h of culture. rhIGFBP-3 and truncated hIGFBP-3 at 10 nM in **a)** (HPLC fractions 36-38) and **b)** (HPLC fractions 39+40); each 3 experiments carried out in triplicate; **c)** Dose dependency of truncated (tr) IGFBP-3 (HPLC fractions 39+40); 2 experiments in triplicate.

cated form. The results (Table I) were similar to those obtained with the rat material: Intact but not truncated BP-3 inhibited IGF I-stimulated DNA synthesis. Inhibition occurred when rhIGFBP-3 doses exceeded those of rhIGF I (Fig. 2). IGFBP-3 was equally effective in blocking the stimulatory effect of

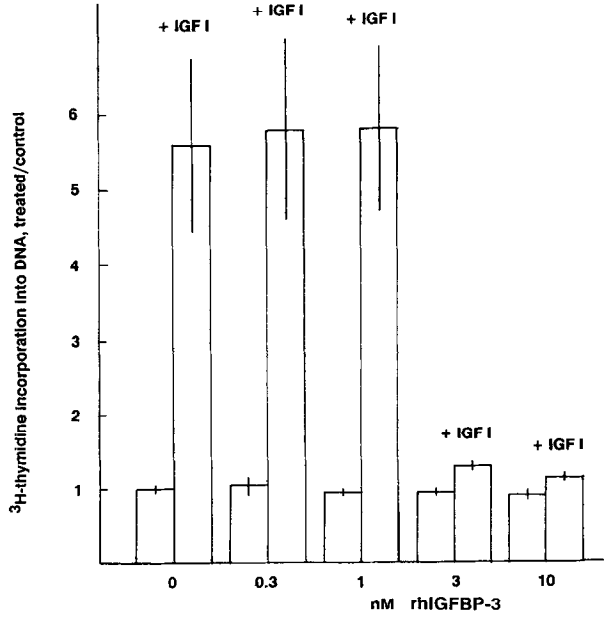

Fig. 2. Inhibition of 1 nM rhIGF I-stimulated DNA synthesis in human osteoblastic osteosarcoma cells by recombinant human IGFBP-3. Values are expressed as treated/control. Columns indicate the mean of n = 6, bars the SEM, from 2 experiments in triplicate.

TABLE II

Stimulation of glycogen synthesis in human osteoblastic (B 10) cells by rhIGF I, insulin, and truncated IGFBP-3 (HPLC fractions 39+40) of rhIGFBP-3

	control	IGFBP-3, 10 nM
control	636 ± 70	645 ± 64
IGF I, 0.1 nM	1040 ± 113	651 ± 74
IGF I, 1 nM	1311 ± 156	663 ± 81
IGF I, 10 nM	1341 ± 127	1553 ± 220
insulin, 5 nM	907 ± 112	983 ± 125
insulin, 50 nM	1171 ± 158	1251 ± 172
tr BP-3, 1 nM	986 ± 123	665 ± 79
tr BP-3, 3 nM	1143 ± 132	704 ± 73

B 10 cells were kept in serum-free F 12 medium for 30 min prior to exposure to fresh F 12-BSA (1 g/l) medium containing test agents and D-U-^{14}C-glucose (1 µCi/dish). Incubations were stopped 4 h later and incorporation of radioactivity into glycogen was measured (13). Data are expressed as ^{14}C-glucose cpm incorporated into glycogen per dish during the 4 h of test incubation (n = 6, mean ± SEM, 2 experiments in triplicates).

IGF II on DNA synthesis in B-10 cells (not shown). Remarkably and different from the observations with truncated rat IGFBP-3, the human preparations had a stimulatory effect on DNA synthesis in the absence of rhIGF I (Table I). HPLC fractions 36-38 (Table I, a) had a moderate, fractions 39+40 (Table I, b) a marked effect. A dose-response curve with fractions 39+40 showed that the latter were 10-fold less potent than IGF I itself (Table I, c).

To estimate the relative proportions between exogenous IGFBPs and the locally produced IGFBPs, ^{125}I-IGF II ligand blot analysis (4) of supernatants was carried out (not shown). It confirmed that the added rhIGFBP-3 was recovered at the molecular mass expected for the nonglycosylated form (33 kDa) (10). After 18 h of incubation, more rhIGFBP-3 and also more of the glycosylated IGFBP-3 (42/45 kDa, produced by the cells) was recovered in supernatants from cells treated with rhIGF I than in supernatant from control cells. This observation is consistent with the hypothesis that IGFs can increase the half life of IGFBP-3. Truncated IGFBP-3, which is less well detectable by ligand blot analysis than full-length IGFBP-3 (8), was not found in cell culture supernatants. Again, effects of 10 nM truncated IGFBP-3 closely resembled those exerted by 1 nM rhIGF I in that comparable stimulation of net IGFBP production was observed (not shown).

To check the specificity of the stimulatory effects, glycogen synthesis was measured. This parameter is rapidly stimulated by IGF I and insulin (13; Table II). IGF I was more potent than insulin in stimulating glycogen synthesis in B-10 cells. The effects of IGF I but not those of insulin were inhibited by rhIGFBP-3 doses exceeding those of the hormones. Truncated IGFBP-3 was also active, and its effect was blocked by rhIGFBP-3.

The consistency with which IGF I effects were mimicked by truncated IGFBP-3 suggested that HPLC fractions 39+40 contained IGF I. Therefore, the preparation used in the experiments of Table I and II was analyzed by SDS-20% PAGE, blotting onto nitrocellulose, and incubation with IGF I antiserum. The data (J.Z., unpublished observation) revealed that fractions 39+40 indeed contained IGF I, as expected from the bioassays, i.e. at a molar ratio of truncated IGFBP-3 to IGF I of about 10 to 1. Furthermore, rhIGF I chromatographed on the HPLC column under the conditions used for separation of the affinity-purified BPs eluted in the same fractions as truncated BP-3.

Our studies show that osteoblastic cells exposed to an excess of rhIGFBP-3 do no longer respond to IGF I. Purified truncated rat IGFBP-3 did not inhibit IGF I action. The human preparations had intrinsic biological activity. The latter can be attributed to IGF I which leaks from the IGF I affinity column and coelutes with this IGFBP from the HPLC column. These findings teach us to be cautious when interpreting effects of affinity-purified IGFBPs. The absence of IGFs has to be conclusively demonstrated before stimulatory effects are attributed to IGFBPs. In fact, several recent reports suggested an intrinsic stimulatory activity of IGFBPs on bone cells (14,15). It will, therefore, be important to perform studies with BPs produced by recombinant technology.

IGF I and IGF II are stored in bone (1). Not only IGFs but also IGFBPs are locally produced in bone (2). In the circulation, they prolong the half-lives of IGFs (4). Many studies have addressed IGFBP effects on cells in vitro. Depending on the IGFBP preparation, the cell type, and the specific experimental conditions, inhibitory or stimulatory effects were found. Thus, IGFBP-3 may inhibit or stimulate IGF-I induced growth of cultured fibroblasts (16). There are few reports regarding bone cells. A recent study reported a positive correlation between paracrine/autocrine production of IGFBP-3 and IGF I-stimulated osteoblast growth (17). Our results show that addition of an excess of IGFBP-3 inhibits the stimulation of bone cells by IGF I. By contrast, such an effect was no longer observed with truncated IGFBP-3.

Evidence of enzymatic degradation of IGFBPs in maternal serum during pregnancy has recently been reported by several authors (for review, 18) and attributed to a pregnancy-associated serum protease activity, apparently specific for IGFBP-3. Such proteolytic activity may result in a release of IGFs from IGFBP-3, since the resulting fragments bind IGFs less tightly than intact IGFBP-3. It has been postulated that activation mechanisms, e.g. by proteolytic enzymes, are of importance in regulating the activity of cytokines locally in bone, e.g. of TGF-β or IGFs (19). Our findings that intact IGFBP-3 but not truncated IGFBP-3 inhibits IGF action on bone cells suggest an important regulatory role of the enzymes responsible for this cleavage; local IGFBP-3 proteolysis may be crucial in stimulating cell growth.

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