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EFFECTS OF INSULIN-LIKE GROWTH FACTOR (IGF) BINDING PROTEINS (BPs) -3 AND -6 ON DNA SYNTHESIS OF RAT OSTEOBLASTS: FURTHER EVIDENCE FOR A ROLE OF AUTO-/PARACRINE IGF I BUT NOT IGF II IN STIMULATING OSTEOBLAST GROWTH

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IGFBP-3 and IGFBP-6 were used to study whether both IGF I and IGF II play a role in auto-/paracrine stimulation of rat osteoblast growth. Both IGFBPs decreased basal DNA synthesis in neonatal rat calvaria cells but with different potencies. Consistent with their IGF binding affinities, IGFBP-3 blocked both IGF I- and IGF II-stimulated DNA synthesis, whereas IGFBP-6 preferentially blocked IGF II-stimulated DNA synthesis. These inhibitory effects of the two IGFBPs can be fully explained by the sequestration of IGFs. Because IGFBP-6 preferentially binds IGF II and is much less potent than IGFBP-3 in decreasing basal DNA synthesis in calvaria cells, IGF I but not IGF II appears to be an important auto-/paracrine stimulator of DNA synthesis. © 1995 Academic Press, Inc.

Osteoblasts are target cells of insulin-like growth factors (IGF I and IGF II). IGF I is more potent than IGF II in stimulating synthesis of DNA, RNA, and collagen (1, 2). Osteoblasts express not only type 1 IGF receptors but also IGF I, IGF II, and IGF binding proteins (IGFBP)-1 to -6 (3, 4). Depending on the particular cell type, the relative contribution of locally produced IGF I versus IGF II to cell function may vary. In particular, human bone cells produce more IGF II than IGF I (5) whereas IGF I is the predominant IGF produced by rat bone cells (4, 6). However, IGF II production by postnatal rat osteoblasts has not been reported.

Measuring the accumulation of IGFs in conditioned cell culture medium may underestimate IGF production and turnover because IGFs may be degraded (7). Therefore, neutralizing anti-IGF I antibodies have been used to demonstrate that locally produced IGF I contributes to auto-/paracrine stimula-

Abbreviations: FCS, fetal calf serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; rh, recombinant human; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein.

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tion of rat bone cell growth (8, 9). In order to investigate the potential role of auto-/paracrine IGF II in rat osteoblast growth, we used IGFBP-6, an IGFBP which preferentially binds IGF II and inhibits its stimulatory effects (10).

MATERIALS AND METHODS

Test compounds:
Recombinant human (rh) IGF I and rhIGF II were from Dr. Märki, Ciba-Geigy, Basel, rhIGFBP-3 was a gift from Dr. A. Sommer, Celtrix, Santa Clara, CA. RhIGFBP-6 was expressed in yeast and purified as described elsewhere (10). [$Q^3A^4Y^{15}L^{16}$]IGF I was provided by Dr. M. Cascieri (Merck, Sharp and Dohme, Rahway NJ) (11). Rat IGF I was kindly provided by Dr. Kobayashi, Fujisawa, Osaka, Japan (12) and rat IGF II was a gift from Dr. P. Nissley, NIH, Bethesda MD.

Cells:
PyMS cells, a rat bone-derived cell line (13), were obtained from Drs. A. Lichtler and D. Rowe, University of Connecticut, Farmington USA.
Normal osteoblasts were prepared from neonatal rat calvaria by sequential digestion with collagenase (1, 4). First passage cells were used.

^{3}H-thymidine incorporation into DNA:
Both cell types were plated at a density of 2 x 10^5/35 mm diameter dish (Falcon) in 5 % FCS DMEM (Gibco). After 2 days, near confluent monolayers had formed, and the medium was replaced with serum-free Ham's F 12 medium (Gibco) for 30 min prior to exposure of the cells to F 12 test medium (Gibco) supplemented with 1 g/l BSA. After incubation for 18 h in test media containing IGFBPs and IGFs as indicated, the cells were pulsed with ^{3}H-thymidine (Amersham, 85 Ci/mmol, 1 µCi/dish), for 3 h at 37°C, rinsed with cold PBS, and DNA was precipitated in situ with 2 ml of 10 % TCA. Incorporated radioactivity was measured in a β-counter.

Western blot analysis:
After 18 h of exposure of both cell types to test medium, aliquots of the medium were removed for IGFBP analysis by ^{125}I-IGF I or ^{125}I-IGF II ligand blot (4) or by IGFBP-3 or -6 immunoblot using polyclonal antisera raised in rabbits (14, 15).

Fate of ^{125}I-rhIGFBP-3 and of ^{125}I-rhIGFBP-6:
Cells were exposed to test medium which contained 10^5 cpm ^{125}I-rhIGFBP-3 or ^{125}I-rhIGFBP-6 (Anawa, Wangen, Switzerland) (specific activity ~200 µCi/µg and ~130 µCi/µg, respectively). After 18 h of incubation at 37°C, aliquots of the cell medium and of the cell layer lysed in SDS were subjected to SDS-PAGE to check the distribution, integrity and recovery of the iodinated proteins.

RESULTS AND DISCUSSION

PyMS osteoblastic cells

PyMS cells are very sensitive to IGF I: 1 nM rhIGF I increases ^{3}H-thymidine incorporation into DNA ~8-fold (table 1). RhIGF I and native rat IGF I (which

Table 1. ^{3}H-thymidine incorporation [cpm/dish x 3 h; mean ± SEM] into DNA of osteoblastic PyMS cells exposed to rhIGFBP-3, rhIGFBP-6, rhIGF I and rh or rat IGF II

A) rhIGFBP-3, rhIGFBP-6, rhIGF I and rhIGF II; 3 experiments in triplicate, n = 9

		control	50 nM IGFBP-3	50 nM IGFBP-6
control		124 ± 22	113 ± 22	112 ± 25
rhIGF I	1 nM	1060 ± 80	104 ± 21	571 ± 15
rhIGF II	5 nM	762 ± 52	91 ± 21	123 ± 25

B) rhIGF II, rat IGF II, and IGFBP-6; 1 experiment in triplicate, n = 3

		control		IGFBP-6 (50 nM)	
		rh IGF	rat IGF	rhIGF	rat IGF
control		108 ± 5		102 ± 41	
IGF I	1 nM	875 ± 33		559 ± 28	
IGF II	1	172 ± 17	150 ± 18	98 ± 5	103 ± 4
	2	296 ± 21	244 ± 22	98 ± 4	102 ± 5
	5	627 ± 36	570 ± 27	102 ± 5	105 ± 5
	10	797 ± 36	718 ± 91	115 ± 6	115 ± 8

differ in 3 out of 70 amino acid residues) (12, 16) are equally potent in stimulating DNA synthesis in this cell line (C. S., unpublished). Stimulation by 1 nM rhIGF I was completely blocked by an excess (50 nM) of rhIGFBP-3 but only partly by excess rhIGFBP-6, (table 1A). The potencies of human and rat IGF II (which differ in 5 of the 67 amino acid residues) (17) in stimulating DNA synthesis were comparable but 10-fold less than the potency of rhIGF I (table 1B). Their effects were completely blocked by both 50 nM rhIGFBP-3 or 50 nM rhIGFBP-6 (table 1A and 1B). Neither IGFBP lowered basal ^{3}H-thymidine incorporation (table 1), consistent with a barely detectable IGF production by this cell line (C. S., unpublished). 3 nM IGFBP-3 or 60 nM IGFBP-6 were required to achieve half-maximal inhibition of 1 nM IGF I-stimulated DNA synthesis (13, table 1A).

Since proteolysis of IGFBPs releases inhibition of IGF effects (13), we checked whether IGFBP-3 and -6 were degraded during the incubation. Media were analyzed for the recovery of added rhIGFBPs after 18 h. The concentration of rhIGFBP-3 but not that of IGFBP-6 decreased during the 18 h of incubation (not shown; but identical results as in fig. 1). Thus, IGFBP-6 was not significantly degraded. Similarly, when ^{125}I-rhIGFBP-6 was added, it was completely recovered from the medium as intact tracer after 18 h of incubation (not shown).

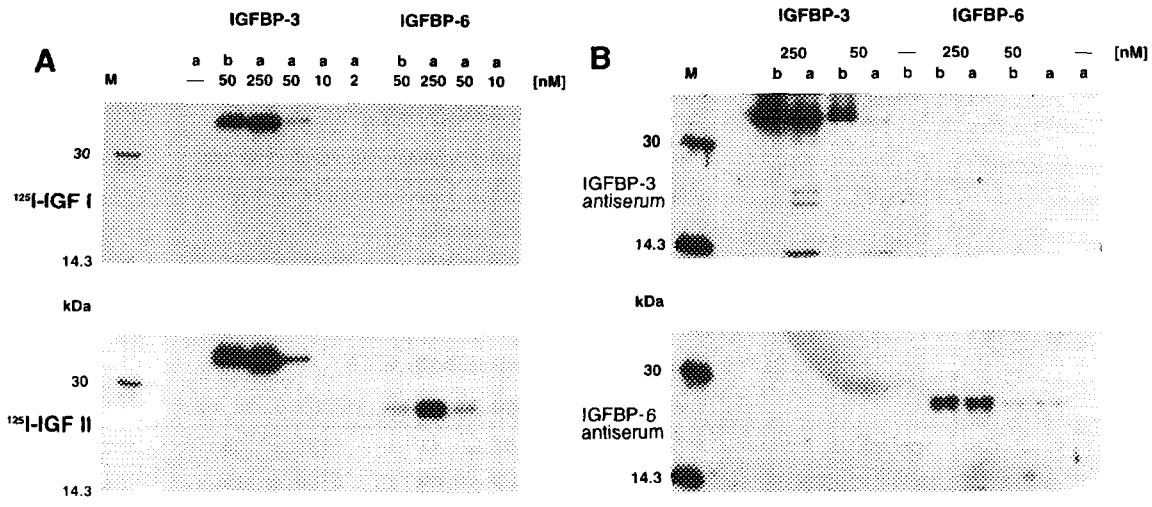

Fig. 1. A. ^{125}I-IGF I and ^{125}I-IGF II ligand blot analysis of rhIGFBP-3 and rhIGFBP-6 containing medium before and after exposure to rat osteoblasts for 18 h. B. IGFBP-3 and IGFBP-6 immunoblot analysis of rhIGFBP-3 and rhIGFBP-6 containing medium before and after exposure to rat osteoblasts for 18 h.

Rat calvaria osteoblastic cells

In contrast to PyMS cells, much higher doses of IGF I and IGF II are required to stimulate ^{3}H-thymidine incorporation into neonatal rat calvaria cells (table 2A and 2B). Maximal stimulation is not even reached at 100 nM (not shown). 5-fold higher concentrations of human or rat IGF II were required to attain similar stimulation as with IGF I (table 2B). Therefore, also higher concentrations of IGFBPs had to be used to block stimulatory effects of added IGFs. In contrast to the findings in PyMS cells, both IGFBP-3 and IGFBP-6 markedly decreased basal DNA synthesis, a finding consistent with significant local IGF production by calvaria cells (4). IGFBP-3 blocked IGF I-stimulated DNA synthesis but not DNA synthesis stimulated by [$Q^{3}A^{4}Y^{15}L^{16}$]IGF I (table 2A), a mutated IGF I with decreased affinity for IGFBPs but normal affinity for type 1 IGF receptors (11). The results of table 2 suggest that IGFBP-3 or a very high concentration of IGFBP-6 decrease DNA synthesis in these cells by sequestering IGF I. IGFBP-6 selectively blocked the stimulation by human and rat IGF II (table 2B) whereas IGFBP-3 blocked the stimulatory effects of both IGF I and (human and rat) IGF II (table 2B). Furthermore, IGFBP-3 was much more potent than IGFBP-6 in decreasing basal DNA synthesis: 10 nM IGFBP-3 was maximally effective (table

Table 2. ^{3}H-thymidine incorporation [cpm/dish x 3 h; mean ± SEM] into DNA of rat osteoblastic calvaria cells exposed to rhIGFBP-3 and rhIGFBP-6, rhIGF I, [QAYL]IGF I, rh and rat IGF II

A) 3 experiments in triplicate, n = 9

		control	IGFBP-3 10 nM	IGFBP-3 100 nM
control		1889 ± 125	1293 ± 116	1038 ± 115
rhIGF I	10 nM	2752 ± 125	2087 ± 145	1187 ± 212
[QAYL]IGF I	10 nM	2763 ± 116	2672 ± 124	2612 ± 136

B) 2 experiments in quadruplicate, n = 8

		control	IGFBP-3 250 nM	IGFBP-6 250 nM
control		1755 ± 52	1191 ± 41	1199 ± 35
rhIGF I	10 nM	2288 ± 79	1408 ± 60	2105 ± 113
rhIGF II	50 nM	2110 ± 112	1180 ± 40	1152 ± 44
rat IGF II	50 nM	2206 ± 83	1354 ± 78	1315 ± 33

C) 3 experiments in triplicate, n = 9

		IGFBP-3	IGFBP-6
0 nM	(control)	1841 ± 57	1841 ± 57
2 nM		1798 ± 126	1928 ± 167
10 nM		1276 ± 76	1793 ± 170
50 nM		1245 ± 47	1434 ± 72
250 nM		1190 ± 73	1247 ± 43

2C) whereas 50 nM IGFBP-6 was required to obtain a significant inhibitory effect. Even higher concentrations were required for maximal inhibition (table 2C).

In contrast to rhIGFBP-6, rhIGFBP-3 was degraded in the medium. Its concentration decreased markedly during the 18 h incubation (fig. 1). In line with their IGF I and II affinities (10), intact IGFBP-3 gave ligand blot signals with both ^{125}I-IGF I and ^{125}I-IGF II, IGFBP-6 only with ^{125}I-IGF II (fig. 1A). Fragments of rhIGFBP-3 but not of rhIGFBP-6 were detected by specific antisera (fig. 1B). RhIGFBP-6 (fig. 1) did not decrease or disappear from the medium during 18 h at 37°C (fig. 1). Studies using ^{125}I-IGFBP-6 (not shown) showed that it remained in its intact form in the medium, with a recovery of close to 100 % after 24 h of incubation. Corresponding studies with ^{125}I-rhIGFBP-3 allowed calculation of an apparent half-life of ~15 h. Consistent with the results of fig. 1 and with observa-

tions in cell-free supernatants from these cells (18), ^{125}I-rhIGFBP-3 is cleaved into smaller molecular mass forms (30, 22, and 17 and 14 kDa).

Conclusions

1) As compared to IGF I, IGF II of both human and rat origin is less potent in stimulating thymidine incorporation into DNA of rat osteoblastic cells (tables 1 and 2).
2) IGFBP-6 is an efficient inhibitor of IGF II- but not of IGF I-stimulated DNA synthesis in rat osteoblastic cells (table 1 and 2).
3) IGFBP-6 is stable in the medium whereas IGFBP-3 is degraded to a significant extent (fig. 1).

The latter finding is important because degraded IGFBP-3 no longer blocks IGF-induced stimulation of DNA synthesis (13). Therefore, the potency by which IGFBP-3 decreases basal DNA synthesis in rat calvaria cells may be underestimated as compared to that of IGFBP-6 (table 2C). IGFBP-6 can serve as a tool to effectively and selectively block the actions of rat IGF II. Remarkably, neither IGFBP-3 nor IGFBP-6 appeared to inhibit DNA synthesis directly in the 2 cell types: in the PyMS cells, basal thymidine incorporation was unaffected, and in the calvaria cells, IGFBPs were not inhibitory in the presence of IGF with low IGFBP-binding affinity: stimulation by [QAYL]IGF I was not prevented by IGFBP-3 (table 2A) and stimulation by IGF I was not inhibited by IGFBP-6 (table 2B). Our findings that basal DNA synthesis in calvaria cells is inhibited by IGFBPs are consistent with inhibition of locally produced IGFs. Because IGFBP-6, in contrast to IGFBP-3, interferes with DNA synthesis only at high concentrations and because rat IGF II is 5-10 times less potent in stimulating DNA synthesis than IGF I, locally produced IGF I but not IGF II appears to play a significant growth promoting role in neonatal rat osteoblastic calvaria cells in vitro.

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