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# Production of carrier proteins for insulin-like growth factors (IGFs) by rat osteoblastic cells

## Regulation by IGF I and cortisol

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A bone-derived rat cell line, PyMS, releases IGF I and IGF carrier proteins which are similar to those found in rat serum. Western blot analysis of culture media conditioned by hormone-treated cells shows that growth hormone and IGF I stimulate and cortisol inhibits production of IGF carrier proteins in vitro. A glycosylated carrier protein species of 49–42 kDa is closely related to the subunits of the growth hormone-dependent carrier protein complex found in rat serum. In addition, rhIGF I rapidly induces a 32 kDa, non-glycosylated IGF-binding protein whose accumulation is markedly increased by cortisol.

Insulin-like growth factor I; Carrier protein; Cortisol; (Bone cell)

## 1. INTRODUCTION

Insulin-like growth factors (IGFs) are structural homologues of insulin affecting growth and differentiation of connective tissue cells both in vivo and in vitro [1]. In serum and other extracellular spaces of the mammalian organism, IGFs are bound to specific carrier proteins [2,3]. The predominant species of carrier protein found in native rat serum is a complex of 150–200 kDa (by neutral gel filtration on Sephadex G-200) consisting of glycosylated subunits with apparent molecular masses of 42–49 kDa (by gel electrophoresis) [3]. While amino-terminal amino acid and cDNA sequences of some IGF carrier protein species have recently been reported [4–7], their physiological roles remain a matter of speculation. Observations that target cells for IGFs, including fibroblasts [8,9] and bone cells [10], produce IGF carrier proteins in vitro suggest that they may regulate the local bioavailability of IGFs. To this end, it appears essential that carrier protein release be well controlled. Therefore, we have studied IGF carrier protein production in a bone-derived rat cell line, PyMS, under the influence of three hormones: IGF I, growth hormone, and cortisol. Our data show that carrier protein release by osteoblastic cells into the culture medium is under endocrine control.

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## 2. MATERIALS AND METHODS

### 2.1. *Cell cultures*

PyMS cells [11] were a generous gift from Dr A. Lichtler, University of Connecticut. This bone-derived cell line was used for the present study since it had been previously found to respond to IGF I and cortisol in a manner comparable to osteoblast-like calvaria cells in primary culture (regarding glycogen, collagen and DNA synthesis). PyMS cells were grown in Falcon dishes (10 cm diameter) to confluence in Ham's F12 medium containing 2 mM glutamine and 1% FCS (all from Gibco), then rinsed with protein-free medium and exposed to test medium as indicated. In order to minimize interference of added IGF carrier proteins, care was taken when supplementing the medium with proteins: it contained either serum from hypophysectomized rats at 0.1% or charcoal-treated bovine serum albumin (BSA, from Serva) at 1 g/l. Hydrocortisone and



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tunicamycin were from Sigma, recombinant human growth hormone from Nordisk and recombinant human (rh) IGF I from Ciba-Geigy. After incubating the confluent monolayers at 37°C in a humidified atmosphere gassed with a mixture of air and $CO_2$ (19:1), the medium was removed and saved for carrier protein analysis.

2.2. *Medium processing*

Comparison of conditioned media with control media without cells allows determination of net IGF carrier protein output by cells in vitro. The collected media were dialyzed against 0.1 M ammonium acetate, lyophilized, and dissolved in 200 $\mu$l distilled water for Western blots and radioimmunoassay.

2.3. *Western blots of carrier proteins*

IGF carrier proteins were identified according to Hossenlopp et al. [2] as described elsewhere [5]. In brief, aliquots of 10 $\mu$l were electrophoresed on 15% SDS-polyacrylamide gels under non-reducing conditions (except the $^{14}$C-labeled molecular mass marker: Rainbow marker, Amersham, England) and transferred to nitrocellulose. The air-dried filters were washed in Tris-buffered saline (TBS; 0.15 M NaCl, 10 mM Tris, pH 7.4) containing 0.1% Tween 20, then incubated with $^{125}$I-labeled IGF II ($4 \times 10^6$ cpm per plastic bag) for 6 h at room temperature. After washing with TBS containing 0.1% Tween 20 the filters were exposed for 1–2 days at −80°C to an X-ray film (Kodak X-OMAT AR) in cassettes equipped with intensifying screens.

2.4. *Digestion of samples with* N*-glycanase*

Aliquots of medium samples were incubated in the presence of *N*-glycanase (Genzyme Corporation, Boston, MA) as described elsewhere [5], electrophoresed and transblotted as above.

2.5. *IGF I radioimmunoassay*

irIGF I was determined [3] using a rabbit antiserum against human IGF I with human IGF I as a standard. Sample aliquots were acid-treated and run on Sep-Pak $C_{18}$ cartridges. They were assayed at 3 different dilutions.

2.6. *Dot hybridization analysis for type I procollagen mRNA*

After washing with cold PBS, total RNA was prepared after extractions with phenol/chloroform and guanidine hydrochloride, dotted in serial dilutions onto nitrocellulose and hybridized to a nick-translated cDNA specific for the $\alpha_1$ chain of type I procollagen as described elsewhere [12]. Hybridization signals reflect steady-state levels of mRNA encoding the $\alpha_1$(I) chain of type I procollagen (per amount of total RNA).

2.7. *Cell number and alkaline phosphatase activity*

Cells were grown on 35 mm dishes to confluence in Ham's F12 medium supplemented with 1% FCS, then exposed to test medium (BSA at 1 g/l) containing the hormones. 4 days later, monolayers were washed with PBS. For the determination of cell number, cells were detached from the dishes with trypsin-EDTA and counted in a hemocytometer. For the determination of alkaline phosphatase activity, cells were lysed into 2 ml of 0.1% Triton X-100 and enzyme activity was assayed in aliquots of cell lysates as in [13] by measuring the cleavage of *p*-nitrophenyl phosphate to *p*-nitrophenol at pH 10.2.

## 3. RESULTS AND DISCUSSION

The rat bone-derived cell line, PyMS [11], was found to release specific IGF-binding proteins into the culture medium. The major species produced by the cells was undistinguishable from the subunits of the large molecular mass carrier protein complex in rat serum [3] (fig.1): $^{125}$I-IGF II detects mainly bands of apparent molecular masses 49/45/42 kDa ('triplet'). The carrier protein species appearing as triplet is more abundant in medium from cells which had been exposed to growth hormone (fig.1). A similar degree of stimulation by growth hormone with a maximum at 1 nM was found in experiments where the test medium contained 1 g/l BSA instead of 0.1% serum from hypophysectomized rats. *N*-Glycanase treatment of medium samples reduced the size of the 49/45/42 kDa bands to yield a single band of 37 kDa (fig.1). Thus, this carrier protein shares at least 4 properties with the subunits which belong to the 200 kDa carrier protein complex, which is the most abundant form in rat serum: IGF binding, molecular size, glycosylation, and growth hormone dependence. However, the amino acid sequence of the protein produced by the cells has not yet been determined, whereas the amino acid sequence of the N-term of proteins from rat serum which yield the triplet on Western blot is known [5].

Tunicamycin, which inhibits glycosylation, completely prevented (at 1 $\mu$g/ml) the release of this carrier protein by the cells. Instead, another IGF-binding protein was found in the medium, the size of which could not be reduced upon treatment with *N*-glycanase.

An experiment addressing the time course of carrier protein release in the absence and in the presence of human IGF I (fig.2) shows accumulation of IGF carrier proteins in the medium, and, in addition, a change in pattern. rhIGF I particularly stimulates the production of a carrier protein migrating at about 32 kDa which cannot be digested with *N*-glycanase (fig.2).

Additional experiments (not shown) revealed that 8 h of IGF I treatment were sufficient to bring about an increased signal of the 32 kDa band. This protein was not detected in media from control-treated and growth hormone-treated cells. Upon prolonged incubation times (4 days), this non-

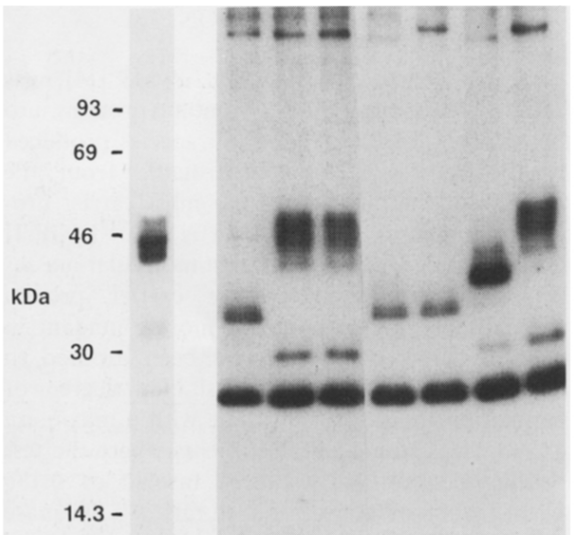


Fig.1. Western blot analysis of IGF carrier proteins in rat serum and in culture media conditioned by rat osteoblastic cells during 4 days. Normal rat serum (RS) and media were analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions, transfer to nitrocellulose, and incubation of blots with $^{125}$I-IGF II for detection of IGF carrier proteins by autoradiography as described in section 2. Cells were exposed for 4 days to MEM/F12 (1:1) medium containing 0.1% serum from hypophysectomized rats. Growth hormone (GH) at 1 nM and tunicamycin (TN) at 1 $\mu$g/ml were added as indicated. The medium samples run on the first 2 lanes were also treated with *N*-glycanase (right, GL).

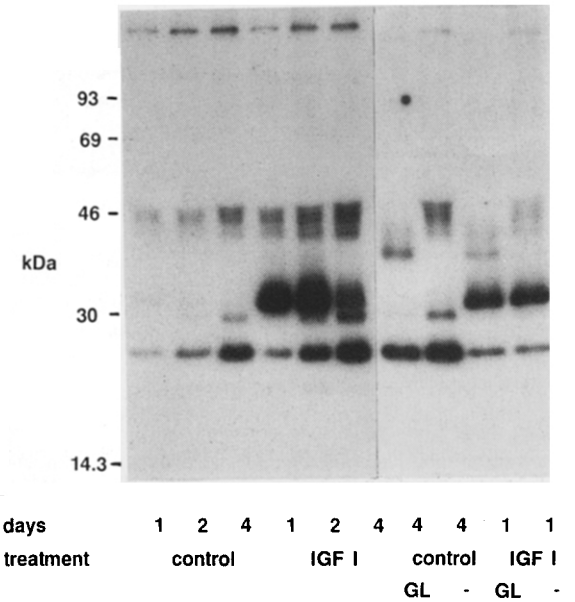


Fig.2. Western blot analysis of IGF carrier proteins in culture media from rat osteoblastic cells after 1, 2, and 4 days of exposure to human IGF I. Cells were exposed to MEM/F12 (1:1) medium for 1, 2, and 4 days, as indicated, in the absence (control) or in the presence of rhIGF I at 10 nM (IGF I). The samples run on lanes 3 and 4 were also treated with *N*-glycanase (right, GL).

glycosylated protein tended to disappear (figs 2,3). Exposure of cells (including pretreament schedules of 2 days) to cortisol still allowed IGF I to induce the 32 kDa band, and, in addition, favored its accumulation in medium conditioned during 4 days (fig.3). As with short term incubations in the absence of cortisol, experiments with a test period of 4 days in the presence of cortisol (1 $\mu$M) yield a dose-dependent stimulation of the 32 kDa band by IGF I, IGF II in higher concentrations and insulin. The relative potency of the 3 hormones is in keeping with their affinity for the type 1 IGF receptor. Again, this effect is not shared by growth hormone. Altogether, the data suggest that this non-glycosylated 32 kDa IGF carrier protein is specifically induced by IGF I and has a short half-life in vitro. A similar carrier protein was transiently present in serum from IGF I-treated hypophysectomized rats [3]. It remains to be seen whether this carrier protein species is related to the 'fetal' carrier protein [4].

It took longer incubation times for the 42–49 kDa carrier proteins (the triplet) to accumulate in the medium, and finally, to become the predominant form (fig.2). As discussed above,

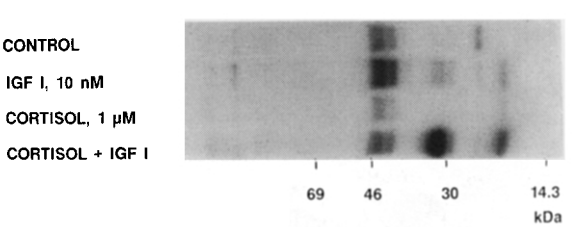


Fig.3. Western blot analysis of IGF carrier proteins in culture media from rat osteoblastic cells exposed for 4 days to cortisol and to insulin-like growth factor I. Cells were exposed for 4 days to F12 medium containing BSA (1 g/l) and hormones, as indicated. Conditioned media were processed as described in section 2. By the same analysis, IGF carrier proteins were not detected in medium which had not been conditioned by cells.

this glycosylated species (figs 1,2) corresponds to the subunits of the growth hormone-dependent large molecular mass carrier complex, the most abundant IGF-binding protein in rat serum [3]. Similarly to the situation in vivo [3], this carrier protein species is found in increased amounts in conditioned medium of cells exposed to IGF I (figs 2,3) or growth hormone (fig.1).

The appearance of this protein in medium from cells which were not stimulated by any hormone may be due to endogenously produced IGF I. As measured by radioimmunoassay, IGF I accumulated in the medium conditioned by cells exposed to hormone- and protein-free medium (experiment shown in fig.2, left): 19 ng human RIA equivalents per dish after one, 14.2 ng after 2, and 54.7 ng after 4 days of culture.

The triplet signal was markedly reduced in medium from cortisol-treated cells and increased in medium from IGF I-treated cells (fig.3). Stimulation of triplet production by IGF I was dose-dependent (with the steepest part of the dose-response curve between 1 and 10 nM), and so was the suppression of the signal by cortisol. Cortisol inhibits DNA and collagen synthesis, and it markedly decreased carrier protein release by PyMS cells at relatively low concentrations (10–100 nM) and in a dose-dependent manner. Under the conditions used in the experiment of fig.3, IGF I increased the relative abundance of type I procollagen mRNA and prevented the decrease caused by cortisol (table 1); likewise, cortisol resulted in decreased alkaline phosphatase activity which was prevented in part by IGF I (table 1).

The effects of IGF I and of cortisol on the release of glycosylated IGF carrier proteins correlate with their effects on DNA synthesis and collagen mRNA levels in the same cells. Reminiscent of the situation in vivo, where the abundance of the subunits belonging to the large carrier protein complex reflects the IGF I status [3], the prevalence of the 49/45/42 kDa bands (triplet by Western blot) in the culture medium appears to correlate also with cell growth in vitro.

In conclusion, the data show that an osteoblastic cell line, PyMS, produces IGF carrier proteins in a controlled manner. This particular cell line was derived from rat calvarial bone cells by introduction of immortalizing DNA sequences, and it has remained responsive to IGFs, cortisol, and growth hormone.

Table 1

RNA yield, $\alpha_1$(I) procollagen mRNA levels, cell number, and alkaline phosphatase activity of rat osteoblastic cells 4 days after exposure to hormones

| Medium | RNA yield ($\mu$g/dish) | Collagen mRNA (treated/control) | Cell number ($10^5$/dish) | Alkaline phosphatase ($\mu$mol/dish per h) |
|---|---|---|---|---|
| Control | 35 | 1.0 | 7.8 ± 0.4 | 4.4 ± 0.2 |
| IGF I, 10 nM | 42 | 2.4 | 8.0 ± 0.3 | 5.6 ± 0.2 |
| Cortisol, 1 $\mu$M | 19 | 0.3 | 5.6 ± 0.5 | 2.0 ± 0.1 |
| Cortisol + IGF I | 34 | 1.5 | 7.4 ± 0.3 | 3.5 ± 0.2 |

After removal of the medium (experiment as in fig.3), RNA was prepared, fixed onto nitrocellulose in equal amounts (2, 1, 0.5 $\mu$g), and hybridized to a nick-translated cDNA specific for the $\alpha_1$ chain of type I procollagen. Hybridization signals were estimated by liquid scintillation counting of cut filters. Relative $\alpha_1$(I) collagen mRNA levels are given as ratios cpm(treated)/cpm(control) per amount of total RNA dotted. To detect hormone effects on cell number and alkaline phosphatase activity under the same conditions, PyMS cells were grown on 35 mm dishes as described in section 2.7. Enzyme activity is expressed as *p*-nitrophenol released. Data are expressed as mean ± SE, $n$ = 8, 2 experiments

Thus far, it has not been possible to directly test [14,15] the effects of native, physiologically occurring IGF carrier proteins on the interaction between IGF I and its receptors on bone cells. Synthesis of IGF carrier proteins by target cells suggests additional auto-/paracrine control mechanisms: bone cells may by hormonally controlled release of IGF carrier proteins determine the availability of IGFs within their own compartment (i) by regulating the amount of IGFs kept in the close extracellular spaces and (ii) by changing (either enhancing or inhibiting) the access of IGFs to their receptors on bone cells.

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