1α,25-Dihydroxyvitamin D_3 increases IGF binding protein-5 expression in cultured osteoblasts

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Abstract Rat osteoblasts produce insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs). Expression of IGFBP-5, an IGFBP which stimulates DNA synthesis of osteoblasts, was studied in vitro under the influence of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and parathyroid hormone (PTH). These two calcium-regulating hormones stimulated the expression of IGFBP-5 mRNA in cultured rat osteoblasts at low concentrations (10 pM) and in a dose- and time-dependent manner. Intact IGFBP-5 was not detected in the culture medium, but was found attached to the cell layer. IGFBP-5 may thus direct IGFs to the sites of bone remodeling.

Key words: 1,25-Dihydroxyvitamin D_3; Parathyroid hormone; Insulin-like growth factor I; Insulin-like growth factor binding protein-5; Insulin-like growth factor binding protein-3

1. Introduction

Insulin-like growth factors (IGFs) favor replication, differentiation and matrix production of osteoblasts [1,2]. IGFs can reach the bone compartment via the blood stream, and they are also synthesized locally. Besides IGF receptors, osteoblasts express IGFs and IGFBPs [3].

Cultured rat osteoblasts produce mainly IGF I [4] and release IGFBP-2 and IGFBP-3 into the medium [5,6]. IGFBP-5 was discovered in the supernatant of human osteosarcoma cells [7] and in extracts of human bone [8], and was found to stimulate DNA synthesis in mouse osteoblastic cells [7–9]. IGF I, prostaglandin E_2, and parathyroid hormone increase IGFBP-5 mRNA in cultured perinatal rat calvaria-derived cells [6,10]. In contrast to IGFBP-3, IGFBP-5 is preferentially bound to extracellular matrix in human fibroblast cultures [11]. Intact IGFBP-5 associates with mouse bone matrix [12].

We have studied the effects of $1,25(OH)_2D_3$ and compared it with the effect of PTH on IGFBP-5 mRNA and on IGFBP-5 in the conditioned culture medium and the cell layer of rat osteoblast cultures because calcium-regulating hormones may use IGF to mediate part of their effects on bone turnover.

2. Materials and methods

2.1. Reagents

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 1α-hydroxyvitamin D_3 ($1\alpha(OH)D_3$), 25-hydroxyvitamin D_3 ($25(OH)D_3$), and 24*R*,25-dihydroxyvitamin D_3 ($24R,25(OH)_2D_3$) were kindly provided by Dr. U. Fischer, Hoffmann-La Roche, Basel, Switzerland. Bovine PTH(1–34) was from Bachem, Torrence, CA. Bovine serum albumin (BSA) (Sigma) was treated with charcoal to remove fatty acids and steroid hormones. Guinea pig anti-serum raised against human IGFBP-5 was a generous gift from D.R. Clemmons, University of North Carolina (Chapel Hill, NC) [13].

2.2. Cell culture

Osteoblast-like cells were prepared from calvaria of newborn rats by sequential digestion with collagenase (CLS II, Worthington) [6]. Primary cell cultures were trypsinized and 10^6 cells were plated per 10 cm diameter (55 cm^2 surface) dishes in phenol red-free α-modified minimum essential medium and Ham's F-12 medium (1:1 mixture, both from Gibco, Paisley, UK) containing fetal calf serum (FCS, 2%), 2 mM glutamine and 50 μg/ml of gentamycin. On the following day, cells were rinsed and fresh medium was added as above, except that FCS was reduced to 1%. 2 days later, cells were rinsed and media replaced by phenol red-free α-modified minimum essential medium and Ham's F-12 medium (1:1 mixture) containing 0.2 g/l of BSA, 2 mM glutamine, and 50 μg/ml of gentamycin for 2×2 days prior to test incubations as indicated.

2.3. Processing of culture media, blot analysis for IGFBPs and radioimmunoassay for IGF I

At the end of test periods, the conditioned media (10 ml) were collected, dialyzed against 0.1 M ammonium bicarbonate, lyophilized and dissolved in 150 μl of distilled water for ligand and immunoblot analysis and for IGF I radioimmunoassay.

15-μl aliquots were electrophoresed on 15% sodium dodecyl sulfate (SDS) polyacrylamide gels under nonreducing conditions (except ^{14}C-labeled molecular weight marker; Rainbow marker, Amersham, UK) and transferred to nitrocellulose. The air-dried filters were washed in Tris-buffered saline (TBS; 0.15 M NaCl and 0.01 M Tris, pH 7.4) containing 0.1% Tween 20 and then incubated with ^{125}I-labeled IGF II (4×10^6 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 as described [6].

For immunoblot analysis 30-μl aliquots were loaded per lane. The air-dried filters were washed in TBS pH 7.6 containing 0.1% Tween 20. The first antibody (anti-IGFBP-5) was used at a dilution of 1:1000, the second (anti-guinea pig, horse-radish peroxidase-conjugated, from Anawa, Wangen, Switzerland) at a dilution of 1:500. Bands were detected by enhanced chemiluminescence with RPN 2106 following the prescription of the supplier (Amersham, UK).

IGF I was extracted by Sep-Pak C18 cartridges and determined using rabbit antiserum (1:20 000) against human IGF I and pure rat IGF I (gift from Dr. Kobayashi, Fujisawa, Japan) as a standard [6].

2.4. Processing of cell layers for analysis of IGFBPs

For analysis of cell layer-associated IGFBPs, 1 ml of the Tris and SDS components of Laemmli buffer were added directly to the tissue culture plate and the material collected for subsequent SDS-PAGE (final concentrations for loading: 0.1 M Tris, pH 6.8, 2% SDS, 20% glycerol). 15-μl aliquots were used for ^{125}I-IGF II ligand blot and 30-μl aliquots for immunoblot analysis as described above.

2.5. Extraction of total RNA and Northern analysis

For RNA analysis, cells were rinsed with ice-cold PBS and lysed in guanidinium isothiocyanate for preparation of total RNA and Northern analysis [6]. Human IGFBP-5 cDNA [14] was used for IGFBP-5 hybridization and rat cDNAs for hybridization with IGF I and β-tubulin mRNA [6].

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3. Results

3.1. IGFBP-5 mRNA expression

IGFBP-5 mRNA was detected as a single 6.5 kb transcript by Northern blot analysis (Fig. 1). The relative abundance of IGFBP-5 mRNA was markedly increased in cells exposed to $1,25(OH)_2D_3$ (Figs. 1 and 2) and PTH (Fig. 2). Both, $1,25(OH)_2D_3$ and PTH were effective at a concentration of 10^{-11} mol/l; $1,25(OH)_2D_3$ was maximally effective at 10^{-9} mol/l and PTH at 10^{-8} mol/l (Fig. 2A). The vitamin D analogues $1\alpha(OH)D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$ were also tested at different concentrations and found to be less potent than $1,25(OH)_2D_3$ by two or more orders of magnitude (not shown). Time course experiments showed that $1,25(OH)_2D_3$, in contrast to PTH, had no significant effect on IGFBP-5 mRNA during the first 6 h. Stimulation plateaued at 24 h and persisted over 48 h, in contrast to that of PTH (Fig. 2B).

3.2. IGFBP-5 in medium and cell layers

As assessed by ligand blot analysis, 42/45/49 kDa IGFBPs, formerly identified as glycosylated forms of IGFBP-3 [15], and 31/32 kDa IGFBPs accumulated in the conditioned medium (Fig. 3, upper left panel) [6]. A 32 kDa form has previously been identified as IGFBP-2 [5]. There is a little association of 42–49 kDa IGFBP-3 with the cell layer, but significant amounts of 31/32 kDa IGFBPs are attached to it (Fig. 3, upper right panel). According to the ligand blot analysis, $1,25(OH)_2D_3$ did not affect the release of IGFBPs into the medium. Intact IGFBP-5 was not detected in the medium by immunoblot (Fig. 3, lower left panel). A 31 kDa band detected by ^{125}I-IGF II ligand blot and IGFBP-5 immunoblot analysis was more prominent in the cell layer lysate of $1,25(OH)_2D_3$- and of PTH-treated than of control osteoblasts (Fig. 3, lower right panel), suggesting that this form corresponds to IGFBP-5.

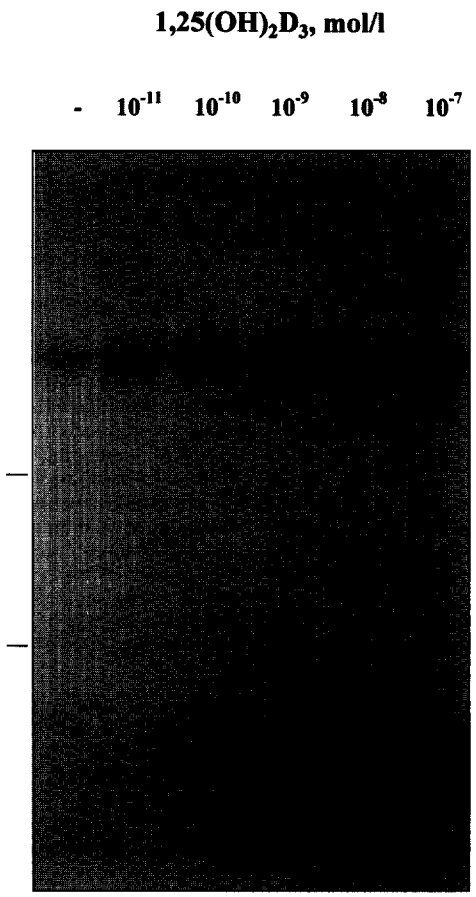

Fig. 1. IGFBP-5 mRNA in rat osteoblasts exposed to $1,25(OH)_2D_3$ for 24 h. Cells were exposed to test media and $1,25(OH)_2D_3$ as indicated, and RNA was prepared after 24 h. 20 μg of total RNA was subjected to Northern analysis. The autoradiograph of the blot after hybridization to cDNA specific for IGFBP-5 is shown.

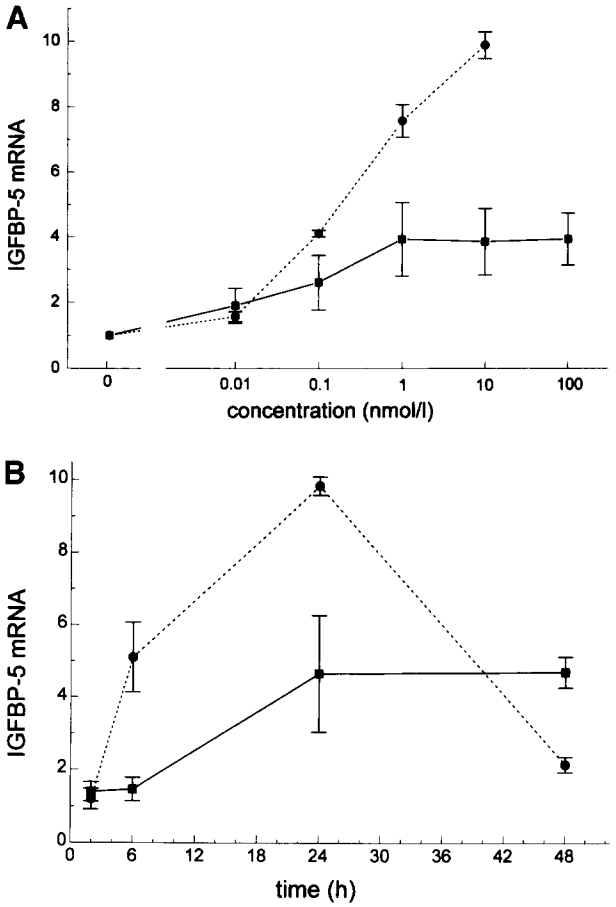

Fig. 2. Effects of $1,25(OH)_2D_3$ and PTH on IGFBP-5 mRNA levels in rat osteoblasts. (A) Dose dependence. Cells were grown as described and exposed to $1,25(OH)_2D_3$ or PTH for 24 h. RNA was prepared and hybridized to cDNAs specific for IGFBP-5 and β-tubulin mRNA. IGFBP-5 mRNA levels are expressed as ratio of optical densities of hormone-treated to vehicle-treated cultures, corrected for optical densities of β-tubulin mRNA signal on the same blot (ratio of optical density of IGFBP-5 mRNA to optical density of β-tubulin mRNA in control cultures is defined as 1). Values represent the mean ± S.D. of 4 experiments. $1,25(OH)_2D_3$ (■——■); PTH (● - - - ●). (B) Time course. Cells were exposed to 5 nM $1,25(OH)_2D_3$ or 5 nM PTH, and RNA prepared after 2, 6, 24, and 48 h was analyzed for the relative abundance of IGFBP-5 and β-tubulin mRNA. For each time point, the optical density of IGFBP-5 mRNA divided by the optical density of β-tubulin mRNA of the control (vehicle-treated sample) was defined as 1. Mean values ± S.D. are given; $n=3$ for time points 2, 6, 24 h, and $n=2$ for 48 h. $1,25(OH)_2D_3$ (■——■); PTH (● - - - ●).

3.3. IGF I production

As evaluated by densitometry (β-tubulin mRNA-corrected),

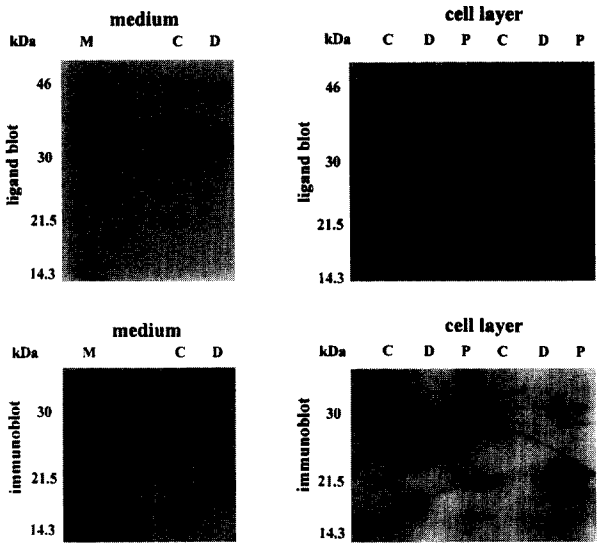

Fig. 3. IGFBPs in serum-free culture media (supernatants, left panels) and in cell layers (lysates, right panels) of cultured rat osteoblasts exposed to test media for 24 h. Upper panels show ligand blots and lower panels immunoblots. Cells were handled and exposed to 10 ml test medium containing 5 nM $1,25(OH)_2D_3$ or PTH for 24 h as described in Section 2. The medium was removed, dialyzed, lyophilized and reconstituted in 150 μl H_2O. 15 μl (i.e. one tenth of the material) was used for ^{125}I-IGF II ligand blot analysis (upper left) and 30 μl (i.e. one fifth of the material) for IGFBP-5 immunoblot analysis (lower left). After washing, cell layers were lysed into 1 ml and 15 μl (i.e. 1.5% of the material) was used for ^{125}I-IGF II ligand blot (upper right) and 30 μl (i.e. 3% of the material) for immunoblot analysis (lower right).

IGF I mRNA levels were 1.5 (mean of 5 experiments; range 0.9–1.9)-fold higher in 5×10^{-9} mol/l $1,25(OH)_2D_3$-treated than in control cells. Analysis of media revealed slightly more IGF I in 5×10^{-9} mol/l $1,25(OH)_2D_3$-treated than in control cultures (range 3.8–15.0 ng/dish) during 24 h of incubation; in 5 independent experiments (duplicate dishes), stimulation was 1.4-fold (range 1.1–1.8-fold).

4. Discussion

The human IGFBP-5 cDNA probe detected a single transcript of 6.5 kb in RNA prepared from rat osteoblasts. The question whether $1,25(OH)_2D_3$ regulates IGFBP-5 and IGF I mRNA expression in normal osteoblasts has not been previously addressed in detail. As shown here, $1,25(OH)_2D_3$ is a potent stimulator of IGFBP-5 mRNA. Its effect appears to be more selective than that of PTH since $1,25(OH)_2D_3$ does not concomitantly stimulate mRNA [6] and release of IGFBP-3 (Fig. 3). In contrast to PTH, $1,25(OH)_2D_3$ has very little effect on IGF I mRNA; similarly, the stimulatory effect of $1,25(OH)_2D_3$ on net release of immunoreactive IGF I into the medium is minor, as previously reported in studies using fetal rat osteoblasts [16]. Therefore, it is unlikely that IGF I mediates the stimulatory effect of $1,25(OH)_2D_3$ on IGFBP-5 mRNA. Moreover, the effects $1,25(OH)_2D_3$ and IGF I on IGFBP-5 mRNA are additive (not shown). Finally, the stimulatory effect of $1,25(OH)_2D_3$, the biologically active metabolite of vitamin D_3, on IGFBP-5 mRNA seems to be specific because the vitamin D_3 analogues were much less potent in stimulating IGFBP-5 mRNA (not shown), consistent with their lower affinities for vitamin D receptors.

IGF I stimulates IGFBP-5 mRNA [6,10]. Both IGF I in the culture medium and IGFBP-5 mRNA increase with time after the preceding medium change in control cells [6]. Therefore, this increase may be due to stimulation of IGFBP-5 mRNA expression by auto/paracrine IGF I. By contrast, IGFBP-3 mRNA does not markedly change with time after medium change, and IGF I does not increase IGFBP-3 mRNA [6].

PTH does not enhance IGFBP-5 mRNA in normal mouse calvariae [12] but is a potent stimulator in normal rat osteoblasts (Fig. 2). It stimulates IGF I mRNA and IGF I release [6] more rapidly than IGFBP-5 mRNA. Therefore, auto/paracrine IGF I may mediate at least part of the stimulatory effects of PTH on IGFBP-5 mRNA expression. Furthermore, stimulatory effects of $1,25(OH)_2D_3$ and PTH were different in time course (Fig. 2B) and additive (not shown).

Intact IGFBP-5 does not accumulate in the medium of rat osteoblasts but can be identified in the cell layer (Fig. 3). In contrast to IGFBP-3, IGFBP-5 is rapidly degraded in the medium but remains intact when associated with the cell layer (Fig. 3) [17].

IGFBP-5 is unique among the IGFBPs because it binds with high affinity and capacity to the extracellular matrix produced by normal osteoblasts [8,12,17]. It is retained in the bone compartment and thus may serve to trap IGFs within bone. Regulated local production and proteolytic cleavage of IGFBP-5 [17] may allow tight local control of IGF bioavailability in bone. The fact that IGFBP-5 mRNA is upregulated by the osteotropic (resorption-stimulating) hormones PTH and $1,25(OH)_2D_3$ fits the hypothesis according to which IGFBP-5 may help IGFs to serve as factors responsible for coupling bone formation to preceding resorption in vivo.

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