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Regulation of Insulin-like Growth Factors I and II and Their Binding Proteins in Human Bone Marrow Stromal Cells by Dexamethasone

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Abstract Glucocorticoids inhibit the proliferation, but induce the differentiation, of bone marrow stromal cells into osteoblast-like cells. The mechanisms, however, are still conjectural. Since insulin-like growth factors (IGFs) have profound effects on osteoblast growth and differentiation, it is possible that glucocorticoids exert their effects on bone marrow stromal cells in part via regulation of IGFs. Therefore, we analyzed the effects of dexamethasone (Dex) on the expression of IGF I and IGF II in cultured preosteoblastic normal human bone marrow stromal cells (HBMSC). Whereas Dex decreased the concentration of IGF I in the conditioned medium since early in the treatment, the concentration of IGF II was increased progressively as culture period lengthened. As the activities of IGF I and IGF II are regulated by the IGF binding proteins (IGFBPs), we analyzed the effects of Dex on the expression of IGFBPs. Dex increased IGFBP-2 in a time-dependent manner. The increase in IGFBP-2, however, was only to the same extent as that of IGF II at most, depending on the length of treatment. Therefore, the increase in IGFBP-2 would dampen, but not eliminate, the increased IGF II activities. By contrast, Dex decreased IGFBP-3 levels, the latter increasing the bioavailability of IGF II. Although IGFBP-4 mRNA levels were stimulated by Dex, IGFBP-4 concentration in the conditioned medium was unchanged as measured by RIA. IGFBP-5 and IGFBP-6 mRNA levels were decreased by Dex in a time-dependent fashion. IGFBP-5 protein level was also decreased 1–4 days after Dex treatment. IGFBP-1 mRNA was not detectable in HBMSC. These accumulated data indicate that Dex regulates IGF I and IGF II and their binding proteins differentially in normal human bone marrow stromal cells. The progressive increase in IGF II may contribute to Dex-induced cell differentiation. J. Cell. Biochem. 71:449–458, 1998.

Key words: dexamethasone; stromal cells; IGF I; IGF II; IGFBPs

Insulin-like growth factors (IGF) I and II are among the most abundant growth factors synthesized by osteoblasts. Both IGF I and IGF II stimulate osteoblast proliferation and matrix protein expression [Frolik et al., 1988; Hock et al., 1988; Schmid et al., 1989; McCarthy et al., 1989; Wergedal et al., 1990; Canalis et al., 1991b, 1993; Mohan and Baylink, 1991; Linkhart et al., 1996]. The autocrine and paracrine activities of IGF I and IGF II on osteoblasts depend not only on their individual concentration, but also on the concentration of IGF binding proteins (IGFBPs). At least six IGFBPs have been identified [Shimasaki and Ling, 1991]. Both basal and regulated secretion of IGFBPs in osteoblast-like cells is cell-line specific and depends on the developmental stage of the cells studied [Hassager et al., 1992; Birnbaum and Wiren, 1994]. IGFBP-3 and IGFBP-4 are the major IGFBPs secreted by the normal human osteoblasts [Hassager et al., 1992; Durham et al, 1994]. IGFBP-2, -3, -4, and -6 are

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usually considered antiproliferative [Mohan et al., 1989; Feyen et al., 1991; Campbell and Novaak, 1991; Kiefer et al., 1992; Jones and Clemmons, 1995], although IGFBP-3 has been shown to increase the mitogenic effect of IGF I in rat osteoblast-like cells [Ernst and Rodan, 1990]. IGFBP-2 also inhibits IGF I-stimulated collagen synthesis [Feyen et al., 1991]. By contrast, IGFBP-5, which has high affinity for the extracellular matrix [Bautista et al., 1991; Andress and Birnbaum, 1992], potentiates the proliferative effects of IGF I and IGF II, and IGFBP-5 alone also induces the mitogenesis of neonatal rat calvarial cells when bound to matrix [Bautista et al., 1991; Andress and Birnbaum, 1992; Jones et al., 1993]. Excess soluble IGFBP-5, however, has been shown to inhibit IGF I- and IGF II-stimulated DNA synthesis in the human osteoblastic osteosarcoma cell line, Saos-2/B-10 [Kiefer et al., 1992].

Glucocorticoids induce the differentiation of bone marrow stromal cells into cells of the osteoblast phenotype, which are capable of matrix mineralization [Owen and Friedenstein, 1998; Beresford, 1989]. We have previously demonstrated that the expression of osteopontin, bone sialoprotein, and osteocalcin by normal human bone marrow stromal cells (HBMSC) is decreased in the presence of Dex, although matrix is mineralized [Cheng et al., 1994, 1996]. Bone sialoprotein has been shown to initiate hydroxyapatite nucleation, while osteopontin and osteocalcin are generally considered inhibitors of matrix mineralization [Romberg et al., 1986; Gorski, 1992; Hunter and Goldberg, 1993; Boskey, 1995; Ducy et al., 1996]. Although a Dex-induced decrease in the expression of osteopontin and osteocalcin is consistent with the induction of matrix mineralization, the decline in bone sialoprotein may hinder this process. Therefore, we hypothesize that alternate mechanisms may also contribute to the induction of matrix mineralization by Dex. Since IGF I and IGF II have been shown to induce the differentiation of osteoblasts and increase the expression of collagen [Hock et al., 1988; McCarthy et al., 1989; Wergedal et al., 1990; Strong et al., 1991; Canalis et al., 1991b, 1993; Mohan and Baylink, 1991], it is possible that Dex exerts part of its differentiation effects on HBMSC by regulation of IGFs and their binding proteins. Although murine bone marrow stromal cells secrete IGF I and IGF-binding proteins constitutively [Abboud et al., 1991], and glucocorticoids regulate the expression of IGF I and some of the IGFBPs in a variety of osteoblastic cells [Hassager et al., 1992; Mohan et al., 1992; Okazaki et al., 1994; McCarthy et al., 1994], there is no information on the expression of basal IGFs and IGFBPs in HBMSC, or on the regulation of their expression by glucocorticoids. Therefore, we pursued experiments designed to analyze the effects of Dex on IGF I, IGF II, and their binding proteins in HBMSC.

MATERIALS AND METHODS

Materials

Dexamethasone, Ficoll/Hypaque (Histopaque-1077, 1.077 g/cm^3), DME/F-12, α-Minimum Essential Medium (α-MEM), trypsin/EDTA, and fetal bovine serum (FBS) were obtained from Sigma Chemical Co. (St. Louis, MO). Mini RiboSep mRNA isolation kit was obtained from Collaborative Biomedical Products (Bedford, MA). cDNA-encoding human IGF II was kindly provided by Dr. Peter Rotwein (Washington University, St. Louis, MO). cDNAs for human IGFBP-1 to -6 were generously supplied by Dr. Shunichi Shimasaki (The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA). cDNA for human β-actin was obtained from Dr. Bratin Saha (Emory University, Atlanta, GA). Megaprime Labeling kit, Hyperfilms, ECL Western blotting analysis system, and 5′-[α^{32}P]-dCTP (3,000 Ci/mmol, aqueous solution) were from Amersham (Arlington Heights, IL). 100× Denhardt's solution, salmon testes DNA, and 50% dextran sulfate were from 5 Prime→3 Prime, Inc. (Boulder, CO). Formamide was from Oncor, Inc. (Gaithersburg, MD). The radioimmunoassay (RIA) kit for IGF I analysis was from Nichols Institute Diagnostics (San Juan Capistrano, CA). Monoclonal antibody (MAb) against rat IGF II was obtained from Amano International (Troy, VA). Rabbit anti-bovine IGFBP-2 antiserum that cross-reacted with human IGFBP-2 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Hercules, CA). Immobilon-P membranes and Sep-Pak C_{18} columns were from Millipore Co. (Bedford, MA). Collagenase A was from Boehringer Mannheim (Indianapolis, IN). The remainder of the chemicals utilized in the experiments were reagent grades and obtained from Sigma.

Human Bone Marrow Stromal Cell Culture

HBMSC were isolated from human ribs (7 female and 13 male) as described previously [Cheng et al., 1994; Kimoto et al., 1994]. The age of donors ranged from 24 to 83 years (mean ± SEM, 52.1 ± 8.0) for females and from 9 to 77 years (mean ± SEM, 37.9 ± 6.5) for males. Their response to Dex treatment was generally similar regardless of the age, sex, or menopausal status of the patients. All assays were performed on first passaged cells. At 3–5 days after seeding (10,000 cells/cm^2), cells were treated with either ethanol (control) or Dex at the indicated concentration and period of time. The day Dex was added to the media was designated as day 0. Although it is well documented that bone marrow stromal cells have the potential to differentiate into a variety of cell types, including adipocytes, chondrocytes, and osteoblasts [Owen and Friedenstein, 1988; Beresford, 1989], no adipocytes or chondrocytes were detected in HBMSC cultures under the experimental conditions used; and most cells (>90%) stained positive for alkaline phosphatase after Dex treatment [Cheng et al., 1994].

IGF I Radioimmunoassay

HBMSC seeded in P-150 were treated with either ethanol or Dex at various doses and for various periods of time as indicated. At 2 days before the end of incubation, cells were washed and incubated in 30 ml of serum-free medium containing either ethanol or Dex. Conditioned media destined for measurement of IGF I were harvested and stored frozen at −20°C. The cell layer was washed with phosphate-buffered saline (PBS), digested with collagenase (1 mg/ml) and trypsin-EDTA, and cell number determined. For IGF I assay, the conditioned medium was treated with glacial acetic acid to a final concentration of 4% and incubated at room temperature for 30 min. Samples were applied to Sep-Pak C_{18} columns, which had been washed sequentially with 5 ml of water, 5 ml of methanol, 5 ml of isopropanol, and 30 ml of 4% acetic acid. After sample application, the column was washed with 20 ml of 4% acetic acid to remove IGFBPs; IGF I was eluted with 2 ml of 75% ethanol in 0.1 M acetic acid twice. The ethanol/acetic acid eluates were combined, dried in a Speed Vac, and the residues analyzed for IGF I using the RIA kit according to the manufacturer's instructions. The recovery of IGF I by this method was approximately 75%. We also performed IGF I assays after sample acidification with acetic acid (final concentration 1 M) and size-exclusion ultrafiltration with a molecular-weight cutoff of 10,000 (UFP 1LGC, Millipore Corp., Bedford, MA) to separate IGF I from its binding proteins [Bowsher et al., 1991] and obtained similar results. Therefore, only the results obtained by employing the Sep Pak C_{18} column separation method are presented.

IGF II Radioimmunoassay

HBMSC were treated with either ethanol or Dex at 10^{-7} M for 9 days. At 2 days before the end of the experiment, cells were washed and incubated in serum-free medium containing control or test solutions. Conditioned media were harvested and stored frozen until IGF II RIA. Cell numbers were determined after collagenase and trypsin-EDTA digestion. Samples were fractionated by acidification and ultrafiltration as described above and freeze-dried. IGF II was measured by RIA as described previously [Canalis et al., 1991a].

IGFBP Analysis by Western Blot Analysis and Radioimmunoassay

IGFBP-2 protein concentration in the conditioned medium was measured by Western blot analysis. An aliquot of conditioned medium, which was normalized to equal cell number, was subjected to SDS-PAGE under reducing conditions and transblotted to an Immobilon-P membrane. The membrane was blocked with PBS containing 3% BSA and 2% dry milk powder, pH 7.4 (blocking solution) for 1 h, followed by incubation with rabbit anti-bovine IGFBP-2 antiserum in blocking solution (1:2,000) for another hour. The membranes were washed with 0.1% Tween 20 in PBS three times and further incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit IgG in this buffer (1:5,000) for 1 h. After washing three times each with 0.3% Tween 20 in PBS and 0.1% Tween 20 in PBS, IGFBP-2 bands were visualized by chemiluminescence using an ECL kit. To quantitate IGFBP-3, -4, and -5, an equal volume of the conditioned medium was subjected to RIA using respective antibodies as previously described [Nakao et al., 1994; Mohan et al., 1995; Honda et al., 1996]. The resulting values were then normalized to cell number.

Northern Blot Analysis

Poly A$^+$-enriched RNA was isolated using a Mini RiboSep mRNA isolation kit according to manufacturer's instructions. RNA was separated on formaldehyde-containing agarose gels and transferred to nylon membranes. Two to four sets of experiments on cells isolated from different patients were performed for each treatment scheme. The membranes were prehybridized with 40% formamide, 5× SSC (0.75 M NaCl in 75 mM Na_3 citrate, pH 7.0), 10 mM Tris HCl, pH 7.4, 1.25× Denhardt's solution and 125 μg/ml salmon testes DNA at 42°C overnight, followed by hybridization in fresh medium containing the same constituents but with additional 10% dextran sulfate and [^{32}P]-cDNA probe to human IGF II, IGFBP-1 to -6, or β-actin overnight at 42°C. [^{32}P]-cDNAs were prepared using the Megaprime Labeling kit and 5′-[α^{32}P]-dCTP according to the manufacturer's instructions. After two washes with 2× SSC and 0.1% SDS for 15 min at room temperature and a single wash with 0.2× SSC and 0.1% SDS at 52°C, the membranes were exposed to Hyperfilms at −70°C. To reprobe with another [^{32}P]-cDNA, the membranes were stripped in boiling 20 mM Tris HCl buffer, pH 7.4, for 15 min, followed by prehybridization and hybridization. To analyze the intensity of each band on the autoradiogram, the X-ray films were subjected to image analysis using ISS SepraScan 2001 (Integrated Separation Systems, Natick, MA). The intensity of each band was normalized to β-actin.

Statistics

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) or Student's *t*-test.

RESULTS

Effect of Dex on the Expression of IGF I in Human Bone Marrow Stromal Cells

Treatment of HBMSC with Dex decreased IGF I concentrations in the conditioned medium to approximately 15% of the control level after 3 days, an effect which was sustained for 9 days (Fig. 1). In separate experiments, the inhibition of IGF I by Dex was observed to be maximal after 2-day treatment (data not shown). The Dex-induced decrease in IGF I levels in the conditioned medium was dose-dependent with significant decrease observed at 1 nM and maximal decrease occurred at 10^{-7} M (data not shown).

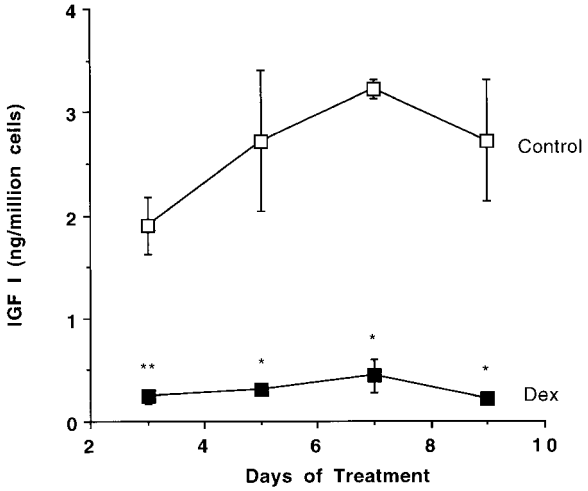

Fig. 1. Inhibition of IGF I by Dex in HBMSC. Cells were treated with either ethanol (control) or Dex at 10^{-7} M for the indicated periods of time. During the last 2 days of treatment, cells were washed and incubated in serum-free medium containing control or test reagent. Conditioned medium was harvested and processed for IGF I by radioimmunoassay (RIA) as detailed under Materials and Methods. Cell layers were washed with phosphate-buffered saline (PBS) and cell numbers counted after collagenase and trypsin-EDTA digestion. Each bar represents the mean ± SEM of three determinations. $^*P < 0.001$, $^{**}P < 0.05$ when compared with the corresponding control value using ANOVA. The data presented are representative of two experiments.

TABLE I. Effect of Dex on IGF II Concentrations in the Conditioned Medium of HBMSC[a]

	IGF II (ng/10^6 cells)	
Treatment	Experiment 1	Experiment 2
Control	5.44 ± 0.91	3.11 ± 0.64
Dexamethasone	19.06 ± 2.57*	10.18 ± 0.8*

[a]HBMSC were treated with either ethanol (control) or Dex (10^{-7} M) for 9 days. During the last 2 days of incubation, the cells were washed and incubated in serum-free medium in the presence of testing agents. Conditioned media were harvested and analyzed for IGF II by RIA. Values shown are means ± SEM of six cultures.
*$P < 0.001$, as compared with the corresponding control value using Student's *t*-test.

Effect of Dex on the Expression of IGF II in Human Bone Marrow Stromal Cells

In contrast to IGF I, Dex increased IGF II concentrations in the conditioned medium 3.5-fold after 9 days (Table 1). Consistent with this observation, Northern blot analysis indicated that Dex progressively enhanced the steady-

state levels of all IGF II mRNA species over a 7-day period (Fig. 2). The relative IGF II mRNA level was 180% of the control value after 1 day and was sixfold of the corresponding control value after 7 days. The enhancement of IGF II mRNA by Dex was dose-dependent with stimulation observed at 10^{-9} M and was maximal at 10^{-8} M (data not shown).

Effect of Dex on the Expression of IGF Binding Proteins in Human Bone Marrow Stromal Cells

Northern blot analysis demonstrated that HBMSC did not express IGFBP-1 either in the absence or presence of Dex (data not shown). Although the control level of IGFBP-2 mRNA was steadily increased as the culture period lengthened to 190% of day-1 level after 7 days, Dex accelerated and further enhanced this increment (Fig. 3, top). The steady-state mRNA level of IGFBP-2 in Dex culture, which was 190% of the corresponding control value after one day, increased fourfold after 7 days. This increment was dose-dependent with stimulation observed at 10^{-8} M and 10^{-7} M (data not shown). Consistent with the increase in IGFBP-2 mRNA by Dex, Western blot analysis indicated that IGFBP-2 (doublets below 46 kDa and partially degraded products in the vicinity of 21.5 kDa) in the conditioned medium increased to 3.5-fold of the control level after 4 days. The stimulation persisted although at a lower magnitude (167% of corresponding control level) after 10 days (Fig. 3, bottom). The increase in IGFBP-2 at the protein level occurred later than that at mRNA level since no difference was observed between the 1-day-treated control and Dex cultures in the Western blot analysis.

In contrast to IGFBP-2, the steady-state mRNA levels of IGFBP-3 were decreased by Dex to 10–20% of the corresponding control

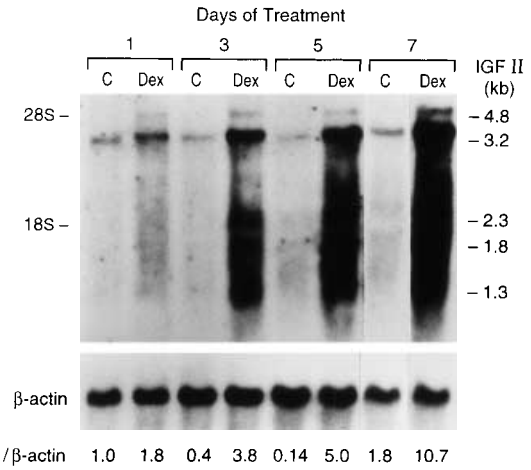

Fig. 2. Effect of Dex on steady-state IGF II mRNA levels in HBMSC. Cells were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days. Poly A^+-enriched RNA preparations were isolated and separated on a formaldehyde agarose gel. The RNAs were transferred to nylon membranes and probed with ^{32}P-labeled human IGF II cDNA followed by ^{32}P-labeled human β-actin cDNA. Expression of IGF II mRNAs (4.8, 3.2, 2.3, 1.8, and 1.3 kb) were visualized by autoradiography. The relative IGF II mRNA concentration on the autoradiograms was analyzed by densitometry and normalized with β-actin. The ratio of IGF II/β-actin for the control cultures after 1 day was defined as 1.0. The data presented are representative of three experiments.

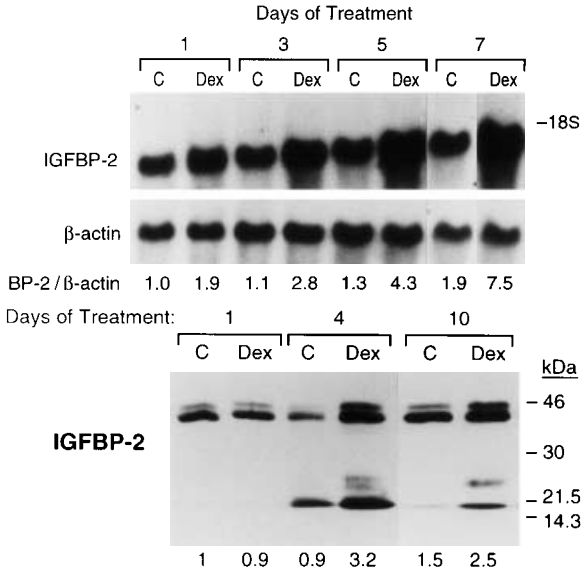

Fig. 3. Effect of Dex on IGFBP-2 expression in HBMSC. Top: Dex effect on the steady-state IGFBP-2 mRNA levels. Cells were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days. Poly A^+-enriched RNA preparations were isolated and Northern blot analysis performed. Membranes were probed with ^{32}P-labeled human IGFBP-2 cDNA, followed by ^{32}P-labeled human β-actin cDNA. Expression of IGFBP-2 mRNA (1.8 kb) was visualized by autoradiography and normalized with β-actin. The ratio of IGFBP-2/β-actin for the control cultures after 1 day was defined as 1.0. The data presented are representative of four experiments. Bottom: Effect of Dex on the protein levels of IGFBP-2. Cells were treated with ethanol (C) or Dex at 10^{-7} M for indicated period of time. At 48 h (except for 1-day treatment, where only 24 h applied) before harvesting, cells were washed with serum-free medium and incubated in this medium together with testing reagent. Conditioned media corresponding to equal cell numbers were separated on SDS-PAGE gel in the presence of reducing agent. IGFBP-2 (doublet near 46 kDa and the partially degraded products adjacent to 21.5 kDa) protein was detected by Western blot analysis. The numbers at the bottom were the relative IGFBP-2 concentration compared with 1-day control culture, which was set as 1. The data presented are representative of two experiments using cells derived from different patients.

values after 1- to 7-day exposure (Fig. 4, top). The control levels of IGFBP-3 mRNA were increased when the culture period lengthened. Likewise, the IGFBP-3 mRNA levels in Dex-treated cultures were increased over 7 days, however, they remained substantially lower than the control levels. The inhibition of IGFBP-3 mRNA by Dex was dose-dependent and detectable at 10^{-9} M (data not shown). Consistent with data obtained from Northern blot analyses, RIA revealed that IGFBP-3 concentration in the conditioned medium was increased continually over 10-day period in the control cultures and Dex prevented this increase (Fig. 4, bottom). After 4- and 10-day Dex exposure, IGFBP-3 concentration in the conditioned medium was 26% and 13% of the corresponding control levels, respectively.

The effect of Dex on IGFBP-4 mRNA was biphasic with initial two- to threefold increments, followed by inhibition to approximately 70% of the corresponding control value after 7 days (Fig. 5). However, the results of RIA testing revealed that Dex had no effect on the IGFBP-4 protein level in the conditioned medium during a 10-day observational period (data not shown). Dex drastically decreased IGFBP-5 steady-state mRNA concentrations after one day and the inhibition persisted for 7 days (Fig. 6, top). Similar to IGFBP-3, IGFBP-5 mRNA levels in the control and Dex-treated cultures were increased as the culture period lengthened, although the levels in Dex cultures remained substantially lower than that of the control cultures. Inhibition of IGFBP-5 by Dex was observed at 10^{-10} M and was maximal at 10^{-8} M (data not shown). Consistent with the Northern blot analysis, Dex suppressed IGFBP-5 protein concentration in the conditioned medium to 15.3% and 33.7% of the corre-

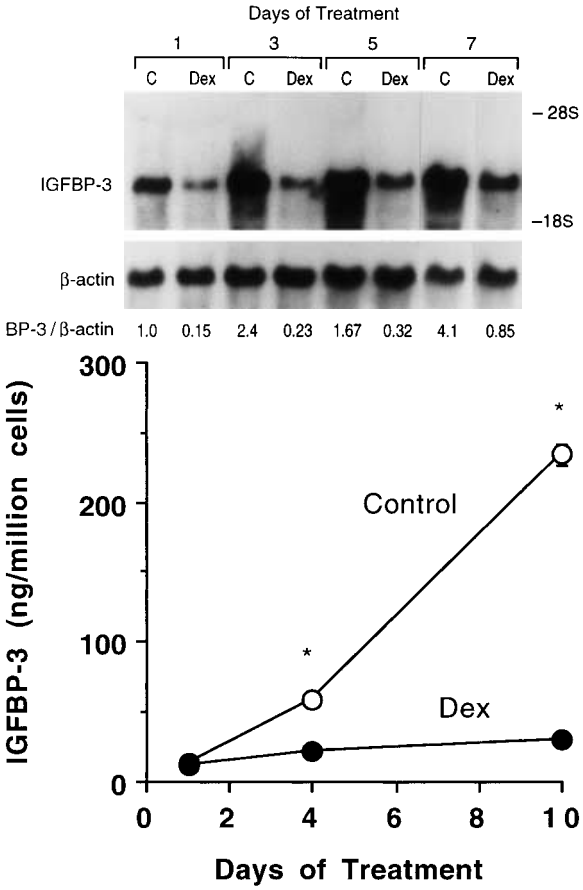

Fig. 4. Effect of Dex on IGFBP-3 expression in HBMSC. Top: Dex effect on the steady-state IGFBP-3 mRNA levels. Cells were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days. Poly A^+-enriched RNA preparations were isolated and Northern blot analysis performed. Membranes were probed with ^{32}P-labeled human IGFBP-3 cDNA, followed by ^{32}P-labeled human β-actin cDNA. Expression of IGFBP-3 mRNA (2.5 kb) was visualized by autoradiography and normalized with β-actin. The ratio of IGFBP-3/β-actin for the control cultures after 1 day was defined as 1.0. The data presented are representative of three experiments. Bottom: Effect of Dex on the protein levels of IGFBP-3. Cells were treated with ethanol (control) or Dex at 10^{-7} M for indicated period of time. At 48 h (except for 1-day treatment, where only 24 h applied) before harvesting, cells were washed serum-free and incubated in this medium together with testing reagent. Conditioned media were subjected to radioimmunoassay (RIA). The concentrations thus obtained were normalized with cell number. The data presented are representative of two experiments. $*P < 0.001$ as compared with the corresponding control value using ANOVA.

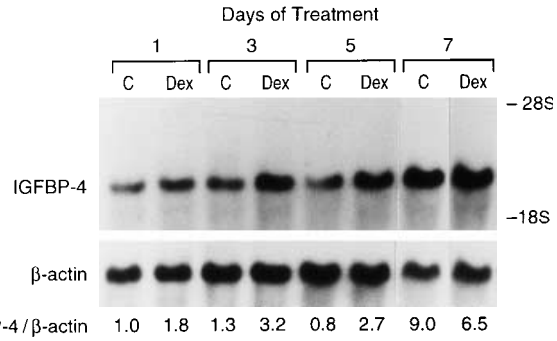

Fig. 5. Effect of Dex on the steady-state mRNA level of IGFBP-4 in HBMSC. Cells were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days. Poly A^+-enriched RNA preparations were isolated and Northern blot analysis performed. Membranes were probed with ^{32}P-labeled human IGFBP-4 cDNA followed by ^{32}P-labeled human β-actin cDNA. Expression of IGFBP-4 mRNA (2.6 kb) was visualized by autoradiography and normalized with β-actin. The ratio of IGFBP-4/β-actin for the control cultures after 1 day was defined as 1.0. The data presented are representative of three experiments.

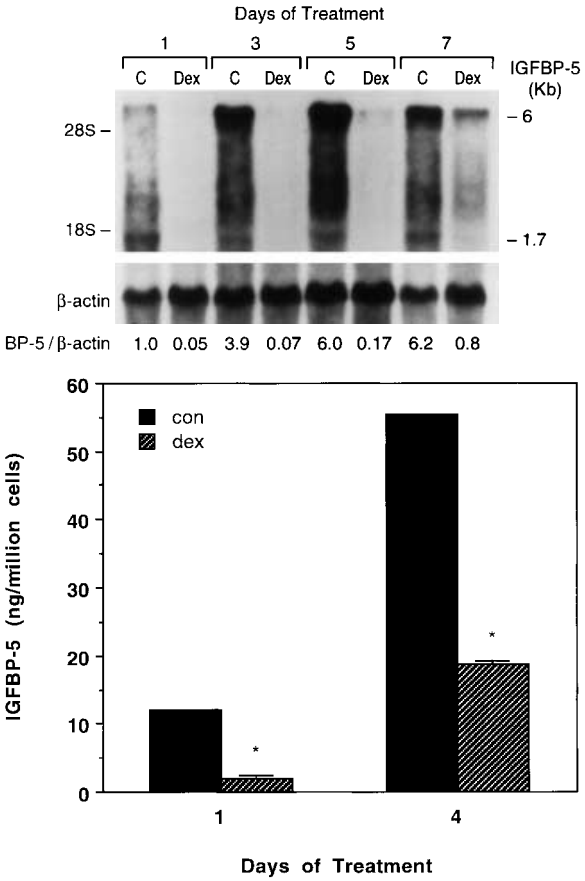

Fig. 6. Effect of Dex on the expression of IGFBP-5 in HBMSC. Top: Dex effect on the steady-state IGFBP-5 mRNA levels. Cells were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days. Poly A^+-enriched RNA preparations were isolated and Northern blot analysis performed. Membranes were probed with ^{32}P-labeled human IGFBP-5 cDNA, followed by ^{32}P-labeled human β-actin cDNA. IGFBP-5 mRNAs (6 kb and 1.7 kb) were visualized by autoradiography and normalized with β-actin. The ratio of IGFBP-5/β-actin for the control cultures after 1 day was defined as 1.0. The data presented are representative of three experiments. Bottom: Effect of Dex on the protein level IGFBP-5. Cells were treated with ethanol (Con) or Dex at 10^{-7} M for 1 or 4 days. At 48 h (except for 1-day treatment, where only 24 h applied) before harvesting, cells were washed with serum-free medium and incubated in this medium together with testing reagent. Conditioned media were subjected to RIA. The concentrations thus obtained were normalized with cell number. The data presented are representative of two experiments. $^*P < 0.001$ as compared with the corresponding control value using ANOVA.

sponding control levels after 1 and 4 days treatment, respectively, although both control and Dex-treated cultures expressed more IGFBP-5 as the culture period increased from 1 to 4 days (Fig. 6, bottom). The decline in IGFBP-5 protein level by Dex was probably not due to proteolysis, since in earlier studies we observed that Dex treatment does not influence IGFBP-5 proteolysis in the conditioned medium of normal human osteoblasts [Chavalley et al., 1996].

The expression of IGFBP-6 mRNA also increased progressively in both control and Dex-treated cultures. Dex, however, had no effect on IGFBP-6 mRNA levels for the first 3 treatment days and only moderately decreased the IGFBP-6 mRNA to 63% of the corresponding control level after 7 days (Fig. 7).

DISCUSSION

Our accumulated results demonstrate that glucocorticoids have profound and differential effects on the expression of IGF I and IGF II and their binding proteins in preosteoblastic HBMSC. Dex decreases IGF I, IGFBP-3, IGFBP-5, and IGFBP-6 levels but increases IGF II and IGFBP-2 levels. Although IGF I is decreased by Dex, the absolute amount of decline in IGF I is far less than the magnitude of increase in IGF II (cf. Fig. 1 with Table 1). Therefore, Dex favors a net increase in IGF (IGF I + IGF II) level. Moreover, the total amount of increase in IGFBP-2 by Dex is at most equal to that of IGF II (cf. Fig. 2 with Fig. 3, top and Table 1 with Fig. 3, bottom). This coupled with the decline in IGFBP-3, IGFBP-5, and IGFBP-6 will further enhance the bioavailability of IGF II in Dex-treated cultures. Although the decrease in IGFBP-5 is consistent with the inhibition of proliferation by Dex, the net increase in IGF II should lead to cell proliferation since IGF II has been shown to be a

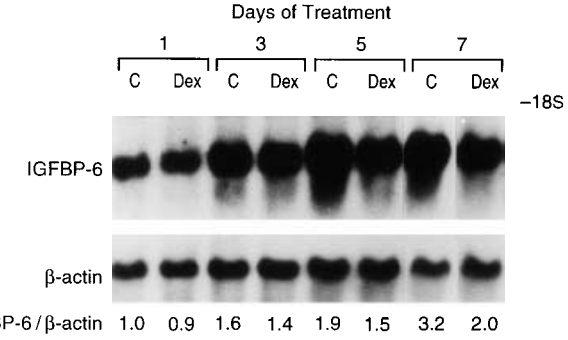

Fig. 7. Effect of Dex on the expression of IGFBP-6 in HBMSC. Cells were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days. Poly A^+-enriched RNA preparations were isolated and Northern blot analysis performed. Membranes were probed with ^{32}P-labeled human IGFBP-6 cDNA, followed by ^{32}P-labeled human β-actin cDNA. IGFBP-6 mRNA (1.3 kb) concentration on the autoradiograms was analyzed by densitometry and normalized with β-actin. The ratio of IGFBP-6/β-actin for the control cultures after 1 day was defined as 1.0. The data presented are representative of four experiments.

mitogen for human osteoblasts [Wergedal et al., 1990; Linkhart et al., 1996]. Therefore, the regulation of IGF and their binding proteins does not appear to mediate the anti-proliferative effect of Dex on HBMSC. Because IGF I protein concentration in the conditioned medium is reduced by Dex, since early in the treatment, the progressive increase in IGF II expression over a 7- to 9-day period (Fig. 2) suggests that IGF II may play more important roles when culture period lengthened. Since it has been reported that IGF II stimulates osteoblast differentiation [McCarthy et al., 1989; Wergedal et al., 1990; Mohan and Baylink, 1991; Strong et al., 1991; Canalis et al., 1993], the differentiation effects of glucocorticoids on osteoblasts may depend at least in part on the increase in IGF II. A reduction of IGF I protein by glucocorticoids has also been reported in rat osteoblasts [McCarthy et al., 1990; Chen et al., 1991b]. The increase in the expression of IGF II at both mRNA and protein levels by Dex, however, is unique to HBMSC, as glucocorticoids have been shown to decrease IGF II levels in nonskeletal cells [Beck et al., 1988; Li et al., 1993] and in intact fetal rat calvaria [Canalis et al, 1991a].

Whereas IGF II concentration is increased in Dex-treated cultures, concomitant increments in IGFBP-2 levels in these cells should blunt, but not totally eliminate, IGF II activity. Although it has not been demonstrated that IGFBP-2 inhibits IGF II activities in osteoblasts, the ability of IGFBP-2 to inhibit IGF I-induced collagen synthesis in osteoblasts [Feyen et al., 1991] suggests that IGFBP-2 could blunt the biological response to IGF II. The increase in IGFBP-2 by Dex in HBMSC is contrary to results obtained in fetal rat calvarial cells, which demonstrate that IGFBP-2 expression is decreased by Dex [Chen et al., 1991a]. HOB, the more mature and differentiated osteoblasts, is reported to express IGFBP-1 but not IGFBP-2 and the level of IGFBP-1 is increased by glucocorticoid treatment [Okazaki et al., 1994]. However, we have observed that IGFBP-2, but not IGFBP-1, is expressed in HBMSC and that IGFBP-2 level is increased by Dex. At present, we are unable to explain the absence of IGFBP-1 in HBMSC, although the differences in the stages of cell differentiation, endogenous growth factor productions, and culture conditions can be contributing factors. The absence of IGFBP-1 in HBMSC, however, is consistent with those observed in fetal rat calvarial cells and murine bone marrow stromal cell lines [McCarthy et al., 1994; Grellier et al., 1995].

IGFBP-3 generally is considered an inhibitory IGFBP in osteoblasts, although a stimulatory effect has been reported in rat osteoblasts [Ernst and Rodan, 1990; Schmid et al., 1991; Andress and Birnbaum, 1992]. The biological effect(s) of IGFBP-3 on IGF in HBMSC is unknown. If IGFBP-3 is inhibitory, the decrease in IGFBP-3 by Dex in long-term treated cells should increase the bioavailability of IGF II, consistent with the differentiation-inducing activity of Dex [Cheng et al., 1994, 1996]. The inhibition of IGFBP-3 production by Dex in HBMSC is similar to previously reported glucocorticoid effects on HOB [Okazaki et al., 1994; Chavalley et al., 1996].

Finally, the maintenance of IGFBP-4 protein level by Dex suggests that this protein does not play important role in mediating Dex effect. By contrast, the drastic decrease in IGFBP-5 and the moderate decrease in IGFBP-6 mRNA by Dex will also increase IGF II bioavailability. It is of note that the transcription of IGFBP-6 was reported to be enhanced by cortisol in fetal rat calvarial cells [Gabbitas and Canalis, 1996], contrary to our observation in HBMSC. In conclusion, dexamethasone modulates differentially the expression of IGF I, IGF II, and their binding proteins in human bone marrow stromal cells with a net increase in IGF II. The increased IGF II may mediate in part the dexamethasone-induced differentiation effects in these cells.

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REFERENCES

Abboud SL, Bethel CR, Aron DC (1991): Secretion of insulin-like growth factor I and insulinlike growth factor-binding proteins by murine bone marrow stromal cells. J Clin Invest 88:470–475.

Andress DL, Birnbaum RS (1992): Human osteoblast-derived insulin-like growth factor (IGF) binding protein-5 stimulates osteoblast mitogenesis and potentiates IGF action. J Biol Chem 267:22467–22472.

Bautista CM, Baylink DJ, Mohan S (1991): Isolation of a novel insulin-like growth factor (IGF) binding protein from human bone: A potential candidate for fixing IGF-II in human bone. Biochem Biophys Res Commun 176:756–763.

Beck F, Samami NJ, Senior O, Byrne S, Morgan K, Gebhard R, Brammar WJ (1988): Control of IGF II mRNA levels by glucocorticoids in the neonatal rat. J Mol Endocrinol 1:R5–R8.

Beresford JN (1989): Osteogenic stem cells and stromal system of bone and marrow. Clin Orthop Rel Res 240:270–280.

Birnbaum RS, Wiren KM (1994): Changes in insulin-like growth factor-binding protein expression and secretion during the proliferation, differentiation, and mineralization of primary cultures of rat osteoblasts. Endocrinology 135:223–230.

Boskey AL (1995): Osteopontin and related phosphorylated sialoproteins: Effects on mineralization. Ann NY Acad Sci 760:249–256.

Bowsher RR, Lee W-H, Apathy JM, O'Brien PJ, Ferguson AL, Henry DP (1991): Measurement of insulin-like growth factor-II in physiological fluids and tissues. I. An improved extraction procedure and radioimmunoassay for human and rat fluids. Endocrinology 128:805–814.

Campbell G, Novaak JF (1991): Insulin-like growth factor binding protein (IGFBP) inhibits IGF action on human osteosarcoma cells. J Cell Physiol 149:293–300.

Canalis E, Centrella M, McCarthy TL (1991a): Regulation of insulin-like growth factor-II production in bone cultures. Endocrinology 129:2457–2462.

Canalis E, McCarthy TL, Centrella M (1991b): Effects of desamino-(1–3)-insulin-like growth factor I on bone cell function in rat calvarial cultures. Endocrinology 129:534–541.

Canalis E, Pash J, Varghese S (1993): Skeletal growth factors. Crit Rev Eukaryot Gene Expr 3:155–166.

Chavalley T, Strong DD, Mohan S, Baylink DJ, Linkhart TA (1996): Evidence for a role of IGF binding proteins in glucocorticoid inhibition of normal human osteoblast-like cell proliferation. Eur J Endocrinol 134:591–601.

Chen TL, Chang LY, Bates RL, Perlman AJ (1991a): Dex and 1,25-dihydroxyvitamin D_3 modulation of insulin-like growth factor-binding proteins in rat osteoblast-like cell cultures. Endocrinology 128:78–80.

Chen TL, Mallory JB, and Hintz RL (1991b): Dexamethasone and $1,25(OH)_2D_3$ modulate the synthesis of insulin-like growth factor-I in osteoblast-like cells. Calcif Tissue Int 48:278–282.

Cheng S-L, Yang JW, Rifas L, Zhang SF, Avioli LV (1994): Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by Dex. Endocrinology 134:277–286.

Cheng S-L, Zhang S-F, Avioli LV (1996): The expression of bone matrix proteins during dexamethasone-induced mineralization of human bone marrow stromal cells. J Cell Biochem 61:182–193.

Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G (1996): Increased bone formation in osteocalcin-deficient mice. Nature 382:448–452.

Durham SK, Kiefer MC, Riggs BL, Conover CA (1994): Regulation of insulin-like growth factor binding protein-4 by a specific insulin-like growth factor binding protein-4 protease in normal human osteoblast-like cells: Implications in bone cell physiology. J Bone Miner Res 9:111–117.

Ernst M, Rodan GA (1990): Increased activity of insulin-like growth factor (IGF) in osteoblastic cells in the presence of growth hormone (GH): Positive correlation with the presence of the GH induced IGF-binding protein BP-3. Endocrinology 127:807–814.

Feyen JHM, Evans DB, Binkert C, Heinrich GF, Gelsse S, Kocher HP (1991): Recombinant human [Cys^{281}]insulin-like growth factor-binding protein 2 inhibits both basal and insulin-like growth factor I-stimulated proliferation and collagen synthesis in fetal rat calvariae. J Biol Chem 266:19469–19474.

Frolik CA, Ellis LF, Williams DC (1988): Isolation and characterization of insulin-like growth factor-II from human bone. Biochem Biophys Res Commun 151:1011–1018.

Gabbitas B, Canalis E (1996): Cortisol enhances the transcription of insulin-like growth factor-binding protein-6 in cultured osteoblasts. Endocrinology 137:1687–1692.

Gorski JP (1992): Acidic phosphoproteins from bone matrix: A structural rationalization of their role in biomineralization. Calcif Tissue Int 50:391–396.

Grellier P, Yee D, Gonzalez M, Abboud SL (1995): Characterization of insulin-like growth factor binding proteins (IGFBP) and regulation of IGFBP-4 in bone marrow stromal cells. Br J Haematol 90:249–257.

Hassager C, Fitzpatrick LA, Spencer EM, Riggs BL, Conover CA (1992): Basal and regulated secretion of insulin-like growth factor binding proteins in osteoblast-like cells is cell line specific. J Clin Endocrinol Metab 75:228–233.

Hock JM, Centrella M, Canalis E (1988): Insulin-like growth factor I has independent effects on bone matrix formation and cell replication. Endocrinology 122:254–260.

Honda Y, Landale EC, Strong DD, Baylink DJ, Mohan S (1996): Recombinant insulin-like growth factor binding protein-4 synthesis of IGFBP-4: Development, validation, and application of a radioimmunoassay for IGFBP-4 in human serum and other biological fluids. J Clin Endocrinol Metab 81:1389–1396.

Hunter G, Goldberg HA (1993): Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci USA 90:8562–8565.

Jones JI, Clemmons DR (1995): Insulin-like growth factors and their binding proteins: Biological actions. Endocr Rev 16:3–34.

Jones JI, Gockerman A, Busby WH Jr, Camacho-Hubner C, Clemmons DR (1993): Extracellular matrix contains insulin-like growth factor binding protein-5: Potentiation of the effects of IGF-I. J Cell Biol 121:679–687.

Kiefer MC, Schmid C, Waldvogel M, Schlapfer I, Futo E, Masiarz FR, Green K, Barr PJ, Zapf J (1992): Characterization of recombinant human insulin-like growth factor binding proteins 4, 5, and 6 produced in yeast. J Biol Chem 267:12692–12699.

Kimoto S, Cheng S-L, Zhang S-F, Avioli L (1994): The effect of glucocorticoid on the synthesis of biglycan and decorin in human osteoblasts and bone marrow stromal cells. Endocrinology 135:2423–2431.

Li J, Saunders JC, Gilmour RS, Silver M, Fowden AL (1993): Insulin-like growth factor-II messenger ribonucleic acid expression in fetal tissue of the sheep during late gestation: Effects of cortisol. Endocrinology 132:2083–2089.

Linkhart TA, Mohan S, Baylink DJ (1996): Growth factors for bone growth and repair: IGF, TGFβ and BMP. Bone 19:1S–12S.

McCarthy TL, Centrella M, Canalis E (1989): Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures. Endocrinology 124:301–309.

McCarthy TL, Centrella M, Canalis E (1990): Cortisol inhibits the synthesis of insulin-like growth factor-I in skeletal cells. Endocrinology 126:1569–1575.

McCarthy TL, Casinghino S, Centrella M, Canalis E (1994): Complex pattern of insulin-like growth factor binding protein expression in primary rat osteoblast enriched cultures: Regulation by prostaglandin E2, growth hormone, and the insulin-like growth factors. J Cell Physiol 160:163–175.

Mohan S, Baylink DJ (1991): Bone growth factors. Clin Orthop Rel Res 263:30–48.

Mohan S, Bautista CM, Wergedal JE, Baylink DJ (1989): Isolation of an inhibitory insulin-like growth factor (IGF) binding protein form bone cell-conditioned medium: A potential local regulator of IGF action. Proc Natl Acad Sci USA 86:8338–8342.

Mohan S, Strong DD, Lempert UG, Tremollieres F, Wergedal JE, Baylink DJ (1992): Studies on regulation of insulin-like growth factor binding protein (IGFBP)-3 and IGFBP-4 production in human bone cells. Acta Endocrinol (Stock) 127:555–564.

Mohan S, Libanati C, Dony C, Lang K, Srinivasan N, Baylink DJ (1995): Development, validation and application of a radioimmunoassay for insulin-like growth factor binding protein-5 in human serum and other biological fluids. J Clin Endocrinol Metab 80:2638–2645.

Nakao Y, Hilliker S, Baylink DJ, Mohan S (1994): Studies on the regulation of insulin-like growth factor binding protein 3 secretion in human osteosarcoma cells in vitro. J Bone Miner Res 9:865–872.

Okazaki R, Riggs BL, Conover CA (1994): Glucocorticoid regulation of insulin-like growth factor-binding protein expression in normal human osteoblast-like cells. Endocrinology 134:126–132.

Owen, M, Friedenstein, AJ (1988): Stromal stem cells: Marrow-derived osteogenic precursors. Ciba Found Symp 136:42–60.

Romberg RW, Werness PG, Riggs BL, Mann KG (1986): Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry 25:1176–1180.

Schmid C, Guler H-P, Rowe D, Froesch ER (1989): Insulin-like growth factor I regulates type I procollagen messenger ribonucleic acid steady-state levels in bone of rats. Endocrinology 125:1575–1580.

Schmid C, Rutishauser J, Schlapfer I, Froesch ER, Zapf J (1991): Intact but not truncated insulin-like growth factor binding protein-3 (IGFBP-3) blocks IGF-I-induced stimulation of osteoblasts: Control of IGF signaling to bone cells by IGFBP-3 specific proteolysis? Biochem Biophys Res Commun 179:579–585.

Shimasaki S, Ling N (1991): Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and –6). Prog Growth Factor Res 3:243–266.

Strong DD, Beachler AL, Wergedal JE, Linkhart TA (1991): Insulinlike growth factor II and transforming growth factor β regulate collagen expression in human osteoblast-like cells in vitro. J Bone Miner Res 6:15–23.

Wergedal JE, Mohan S, Lundy M, Baylink DJ (1990): Skeletal growth factor and other growth factors known to be present in bone matrix stimulate proliferation and protein synthesis in human bone cells. J Bone Miner Res 5:179–186.