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Insulin-Like Growth Factor Binding Proteins in Femoral and Vertebral Bone Marrow Stromal Cells: Expression and Regulation by Thyroid Hormone and Dexamethasone

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Abstract Insulin-like growth factor (IGF)-I is an important regulator of bone metabolism. Clinical observations suggest that different anatomic sites within the adult skeleton respond differently to hormonal and therapeutic treatment, and recent studies on bone marrow stromal cells in culture show that there are skeletal site-dependent differences in the gene expression of IGF-I. The actions of IGF-I and -II on bone cells are known to be modulated by the IGF binding proteins (IGFBP)-1 through -6 and the Type I and Type II IGF receptors. Therefore, we compared the expression of IGFBP-1 through -6 in adult female rat bone marrow stromal cell cultures derived from two separate skeletal sites: vertebrae and femurs. The cultures were maintained simultaneously under conditions that support osteoblast differentiation from osteoprogenitors present in the femoral and vertebral marrow cell populations. We also addressed whether IGFBP messenger RNA levels are regulated by thyroid hormone (T_3) and dexamethasone (dex) treatment in femoral vs. vertebral marrow stromal cells in vitro, since steroid hormones play an important role in skeletal function. Northern blot analyses revealed that there are distinct skeletal site differences in the gene expression of IGFBPs. The vertebral marrow cultures express IGFBP-2 through -6 mRNAs, with IGFBP-2, IGFBP-4, and IGFBP-6 mRNAs predominating. The femoral marrow stromal cell cultures express only IGFBP-4 and IGFBP-6. Importantly, vertebral marrow cultures have much higher IGFBP mRNA steady-state levels than femoral cultures for all the detected IGFBP transcripts. IGFBP-1 is not detected in either femoral or vertebral cultures. In addition to a skeletal site difference, we show that T_3 and dex regulate the expression of specific IGFBP mRNAs. T_3 treatment also upregulates IGF-I protein secretion by vertebral marrow stromal cell cultures. Interestingly, the type I receptor for IGF-I was expressed equivalently in cultures from the two skeletal sites. These findings have important implications for the anatomical site specificities of hormonal responses that are noted in the skeleton. J. Cell. Biochem. 81:229–240, 2001.

Key words: insulin-like growth factor binding proteins; bone marrow stromal cells; thyroid hormone; dexamethasone

Bone marrow stroma as a tissue consists of a complex network of multiple cell types, growth factors, cytokines, and extracellular matrix components. The stromal cell population supports hematopoiesis and osteoclast formation, and also contains progenitors that have the ability to form adipocytes, cartilage, muscle, and bone [Friedenstein et al., 1987; Beresford, 1989; Long and Mann, 1993; Owen, 1998]. The processes of bone formation and resorption in the adult skeleton can be better understood by considering the marrow environment as a whole [Sharrock, 1998; Gehron Robey and Bianco, 1999]. Thus, models have been developed to study the differentiation and function of osteoblasts in bone marrow-derived cell cultures [Maniatopoulos et al., 1988; Cheng et al., 1994; Aubin, 1998]. As a result, marrow cell cultures are now being used to investigate human bone diseases [Bianco and Gehron Robey, 1999]. Future directions include the transplantation of in vitro expanded and genetically manipulated marrow stromal cell cultures to promote bone healing [Frenkel et al., 1997; Prockop, 1998; Hou et al., 1999; Stein et al., 2000].

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Due to their recently acknowledged usefulness for investigating bone biology and disease, results from marrow stromal cell culture studies must be more rigorously interpreted with respect to the variability of osteoblast responses that occur due to species differences, animal age, gender differences, and skeletal site differences. Of the many different local and systemic factors that exert control over bone cell activity, insulin-like growth factors (IGF) I and II are among the most abundant growth factors found in bone. IGFs enhance the differentiated function of osteoblasts and increase bone formation [Canalis, 1997]. Previously, we demonstrated that osteogenic marrow cultures derived from adult rat vertebrae have much higher IGF-I mRNA levels compared to femur-derived marrow cultures, and that the addition of T_3 to the culture medium causes a dose-dependent increase in IGF-I mRNA levels in vertebral marrow cultures [Milne et al., 1998a, 1998b].

Further investigation of IGF action at different skeletal sites must also take into account the expression of the IGF binding proteins (IGFBPs) and the IGF receptors. The IGFBPs constitute a family of six structurally related proteins that bind IGFs with high affinity. The actions of IGF-I are potently modulated by these binding proteins [Rechler and Clemmons, 1998]. Our present study compares the expression of IGFBP-1 through -6, IGF type I receptor, and gene regulation by T_3, in adult rat marrow stromal cell cultures derived from two different anatomical sites: femurs and vertebrae. We also measured IGF-I polypeptide levels in the media conditioned by these cultures.

When adult rat-derived femoral or vertebral marrow cells are maintained in a medium which includes fetal bovine serum (FBS), ascorbic acid, β-glycerophosphate, and dexamethasone (dex), osteogenic differentiation occurs in cultures from each skeletal site, as evidenced by high alkaline phosphatase activity, an upregulation of osteoblast-associated genes, and the formation of three-dimensional nodule structures which mineralize [Milne et al., 1998a]. Under the culture conditions and plating densities used, the presence of glucocorticoid (dex) is essential to support osteoblast differentiation from both skeletal sites [Milne et al., 1998b]. In osteogenic bone marrow cultures, the osteoblasts coexist with multiple adherent marrow cell types which are as yet poorly identified, but most certainly secrete factors that support osteoblast differentiation and bone formation [Aubin, 1999]. Recently, glucocorticoids have been shown to regulate the expression of IGF-I, IGF-II, and IGFBPs in bone marrow stromal cell cultures derived from adult human rib bones [Cheng et al., 1998]. However, no information exists comparing the effects of glucocorticoids on the expression of IGF regulatory components by marrow cells obtained from different anatomical sites in the skeleton. Therefore, in addition to T_3 regulation studies, we also examined the actions of dex on the gene expression of IGF-I binding proteins in cultures of femoral vs. vertebral marrow stromal cells.

METHODS

Cell Cultures

The method used to obtain bone marrow cultures from young adult rats is described in detail previously [Milne et al., 1998a, 1998b]. The femurs and lumbar vertebrae were obtained from Sprague-Dawley female rats (100–125 g). The animals were sacrificed by methods approved by the University of Massachusetts Medical School Animal Care and Use Committee. Reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. All experiments were performed on secondary (first passage) cultures. Cells were seeded onto uncoated 6-well culture plates at 2.5×10^4 cells/ml (6.25×10^3 cells /cm^2) for the T_3 response studies or at 5.0×10^4 cells/ml (1.25×10^4 cells/cm^2) for the dex response studies. The culture medium was αMEM supplemented with 20% fetal bovine serum (FBS lot #7008c; Atlanta Biologicals, Norcross, GA), ascorbic acid (50 μg/ml), β-glycerophosphate (β-GP, 10 mM) and antibiotics.

For T_3 studies, all cells were supplemented with dex (10^{-7}) and either ethanol (control) or T_3 (10^{-8} or 10^{-7} M). The T_3 was present continuously in the culture media, beginning at the time the secondary cultures were seeded (Day 0). Cell layers were harvested for gene expression studies after 6, 9, 12, or 15 days of treatment. Conditioned media was collected on days 9,12, 15, and 18 for IGF-I radioimmunoassay. For the dex response studies, cells were treated with either ethanol (control) or dex (10^{-8} or 10^{-7} M), which was present throughout the culture period (Day 0 through Day 6). Cell

layers were then harvested for RNA isolation and Northern blot analyses.

In a separate experiment, vertebral cultures were serum-deprived in order to measure IGF-I accumulation into the medium in the absence of FBS and to restrict gene expression responses to that of the exogenously added T_3. The procedures of Schmid et al. [1992] were followed to gradually deplete the osteogenic cultures of the serum. Secondary vertebral marrow cultures were maintained for 6 days in media which support osteoblast differentiation (20% FBS, ascorbic acid, β-GP, and 10^{-7} M dex). T_3 (10^{-8} M) or ethanol vehicle was present throughout the culture period. On Day 6 (when cuboidal osteoblast colonies were first visible in the cultures), the FBS concentration was lowered to 5% and was further decreased to 1% on Day 7. On Day 8, serum was replaced with bovine serum albumin (300 mg/100 ml medium) and cultures were maintained serum-free ($\pm T_3$) for an additional 4 days, with no further media changes. Conditioned media samples were then collected from serum-free control and serum-free T_3-treated cultures and stored at −80°C for IGF-I assay. These media samples were also assayed for the presence of IGF binding proteins. The corresponding cell layers were harvested for RNA isolation and gene expression studies.

RNA Isolation and Northern Blots

Total RNA from control, T_3-treated, and dex-treated femoral and vertebral marrow cultures was isolated from duplicate wells by the guanidine thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. The RNA samples (5 μg total RNA per lane) were size-fractionated on 1.2% agarose/1.8% formaldehyde gels, followed by transfer to Nytran Plus membranes (Schleicher and Schuell, Keene, NH). To document equal loading of the RNA samples, ribosomal RNA on the membranes was visualized by ethidium bromide. For the detection of IGF type I receptor transcripts, 20 μg of total RNA per lane were analyzed. All membranes were UV-cross-linked prior to hybridization. Gene probes specific for each of the rat IGF system components were gel-purified inserts from plasmids kindly provided by Dr. E. Canalis (St. Francis Hospital and Medical Center, Hartford, CT). Permission to utilize the cDNA for IGFBP-2 [Nakatani et al., 1991], IGFBP-3 [Shimasaki et al., 1989], IGFBP-4 [Shimasaki et al., 1990], IGFBP-5 [Shimasaki et al., 1991a], and IGFBP-6 [Shimasaki et al., 1991b], was obtained from Dr. S. Shimasaki (School of Medicine, University of California, San Diego). Permission to use the rat cDNA clones for IGF-I [Murphy et al., 1987] and IGFBP-1 [Shimasaki and Ling, 1991c] was obtained from Dr. L. Murphy (University of Manitoba, Winnipeg, Manitoba, Canada). Permission was obtained from Dr. D. LeRoith (NIH, Bethesda, MD) for the use of the IGF-I receptor probe [Werner et al., 1989]. The rat osteocalcin (OC) [Lian et al., 1989], GAPDH [Tso et al., 1985], and collagen type I (Coll I) [Genovese et al., 1984] cDNAs were kindly provided by Drs. J. Lian and G.S. Stein (University of Massachusetts Medical School, Worcester, MA). Rat bone sialoprotein (BSP) cDNA [Fisher et al., 1990] probe was provided by Dr. L. Fisher (NIH, Bethesda, MD). Hybridizations were performed at 42°C for 18 h, with high stringency conditions (50% formamide, 1% SDS, 1 M NaCl) with 10 ng cDNA inserts labeled to high specific activities ($>1 \times 10^8$ cpm/μg) by the random primer method [Feinberg and Vogelstein, 1983]. The membranes were washed at a final stringency of $0.1 \times$ SSPE/0.1% SDS at 55°C and exposed to BioMax MS film with BioMax TranScreen-LE- or -HE-intensifying screens (Eastman Kodak, Rochester, NY). For valid comparison of gene expression levels, femoral vs. vertebral Northern blot membranes were always hybridized together and exposed side by side on the same sheet of film. Densitometric values of autoradiography exposures were determined using Quantiscan software (Biosoft, Cambridge, UK) The membranes were stripped, and the total removal of membrane-bound [^{32}P]-labeled probes was confirmed by autoradiography. The same membranes were subsequently hybridized with the additional cDNA probes. All hormone response studies were performed a total of three times, with consistent results obtained.

IGF-I Protein Measurements

Conditioned media samples were collected from duplicate cell cultures for the time course studies and from triplicate cultures for the serum-free T_3 treatment studies and stored at −80°C until analysis. All IGF-I radioimmunoassays (RIAs) were performed at the Maine Center for Osteoporosis Research, St. Joseph Hospital, Bangor, ME. The media samples

were acid–ethanol-extracted according to the procedures of Brier et al. [1991] to remove IGFBPs that interfere with the IGF-I RIA. Assays were performed in duplicate using a polyclonal antibody and a purchased IGF-I RIA kit (Nichols Institute, San Juan Capistrano, CA).

RESULTS

The Expression of IGF System Components in Osteogenic Bone Marrow Stromal Cell Cultures: Effects of T_3

Northern blot analysis of total RNA from marrow cell cultures analyzed on days 6–15 revealed a striking difference between femoral and vertebral cells with regard to the level of expression of the IGFBPs (Fig. 1). In order to compare gene expression of the IGF system components in parallel with the osteoblast developmental sequence, the cells were cultured under conditions which support osteoblast differentiation from osteoprogenitors present in the femur and vertebrae bone marrow cell populations. The osteogenic culture medium contains dex at a concentration of 10^{-7} M. Consistent with prior observations, cultures derived from both skeletal sites expressed the high OC mRNA levels typical of differentiated osteoblasts.

In vertebral marrow cells, IGFBP-2 mRNA (1.4 kb transcript) was apparent throughout

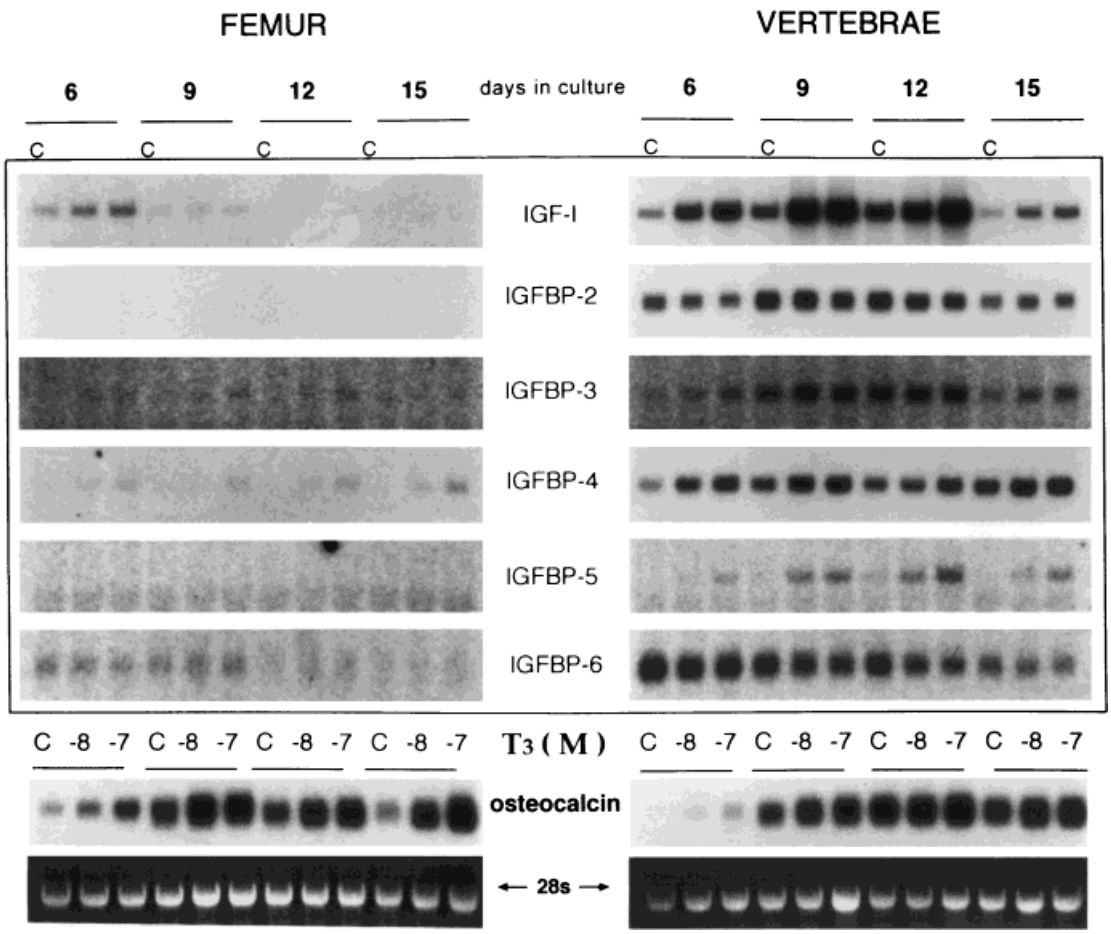

Fig. 1. Time course comparing the relative gene expression of IGF binding proteins in untreated (control) and T_3-treated bone marrow stromal cell cultures. Femoral and vertebral secondary cultures were grown in a medium which supports osteoblast differentiation, with or without T_3, for the indicated number of days. Total RNA was extracted, and 5 μg/lane was used for Northern blot analysis. The blots were sequentially probed together for detection of the IGFBP mRNAs. The membranes were also probed for OC to assess osteoblast gene expression. For each probe, femoral vs. vertebral membranes were exposed on the same film for identical lengths of time: IGF-I (100 h), IGFBP-2 (34 h), IGFBP-3 (140 h), IGFBP-4 (22 h), IGFBP-5 (86 h), IGFBP-6 (31 h), and OC (24 h). The ethidium bromide-stained 28s ribosomal band was used to assess sample loading. The T_3 concentrations used for the IGFBP and OC gene expression studies are shown at the bottom of the figure.

the culture period, and steady-state mRNA levels did not change appreciably with the addition of T_3. IGFBP-3 (2.7 kb) was expressed at low levels in vertebral cultures, and T_3 supplementation dose-dependently increased IGFBP-3 levels (based upon densitometric readings, up to 1.6-fold on Day 15 by 10^{-7} M T_3). IGFBP-4 transcripts (2.3 kb) were abundant in vertebral cultures at all time points examined, and small but consistent increases in mRNA levels were induced by T_3 supplementation. Vertebral cultures had low levels of IGFBP-5 mRNA (6.3 kb) when maintained under osteogenic culture conditions, which include 10^{-7} M dex. However, the addition of T_3 to these cultures dose-dependently increased IGFBP-5 gene expression at each time point examined. IGFBP-5 expression was elevated up to four-fold on Day 12. IGFBP-6 (1.1 kb) was abundantly expressed in vertebral marrow osteogenic cultures, and the T_3 effects on steady-state mRNA levels were marginal.

Northern blot analysis of femoral osteogenic cells revealed that IGFBP-2, -4, and -5 gene expression were undetectable in the control cultures. However, T_3 dose-dependently increased IGFBP-4 mRNA levels. The IGFBP-3 transcript was weakly seen in femoral control cultures on Day 15 only, with T_3 treatment marginally upregulating IGFBP-3 levels. Low levels of IGFBP-6 transcripts were detected in femoral cultures, and T_3 supplementation did not appreciably alter IGFBP-6 steady-state levels. Neither femoral nor vertebral cultures contained detectable levels of IGFBP-1, even when maintained in the presence of T_3 (Northern blots not shown).

Consistent with our previous findings [Milne et al., 1998a, 1998b], vertebral osteogenic cultures had much higher steady-state IGF-I mRNA levels than femoral cultures (Fig. 1). T_3 caused a dose-dependent increase in IGF-I expression in vertebral cells at all time points examined. Femoral cultures expressed IGF-I early in the culture period, with expression diminishing with time in culture. T_3 caused an elevation in femoral IGF-I expression, which was evident on Day 6.

The stimulatory effect of T_3 on vertebral IGF-I gene expression was reflected in an increased accumulation of IGF-I protein in the culture medium (Fig. 2). T_3 addition (10^{-8} and 10^{-7} M) increased IGF-I protein levels to a maximum of 1.6-fold in the conditioned media collected on Day 18. Thus, protein secretion was highest in mineralizing vertebral cultures, even though IGF-I mRNA levels at this time point had declined. In a culture medium (20% FBS) not conditioned by cells, IGF-I levels were 13.7 ng/ml. In the medium conditioned by control or T_3-treated femoral cultured cells, IGF-I protein levels did not exceed the basal level contributed by the 20% serum (Fig. 2).

As shown in Figure 3, a cDNA probe for the rat IGF-I receptor gene detected transcripts of ≈ 11 kb present in RNA derived from femoral and vertebral cultures. The cells had been

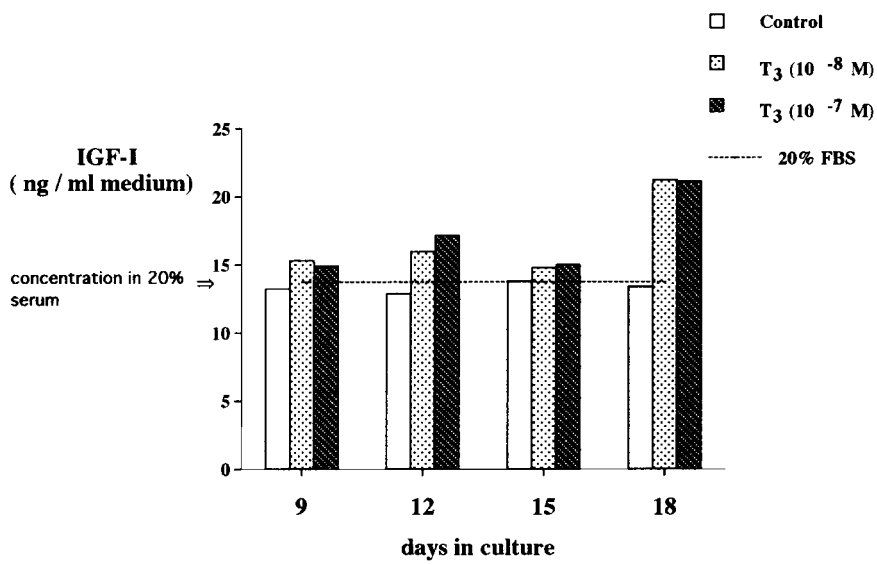

Fig. 2. IGF-I protein secretion in the media of vertebral marrow stromal cell cultures. Secondary osteogenic bone marrow cultures were treated with vehicle (control) or T_3 for the indicated number of days. Data points show the average of duplicate wells per treatment.

maintained for 6 days under osteogenic culture conditions, in the presence of vehicle or T_3 (10^{-8} M). There was no marked difference in the levels of expression of IGF-I receptor in cultures from the two skeletal sites. No T_3-dependent changes were observed.

Expression of IGF System Components in Serum-Free Vertebral Cultures: Effects of T_3

In order to evaluate the direct effect of T_3 addition on IGFBP gene expression and to also investigate IGF-I production in the absence of serum, vertebral marrow cells were gradually deprived of FBS once the morphological appearance of osteoblasts was visible in culture (Day 6). Control and T_3-treated cells were harvested for RNA analysis on Day 12, and conditioned media samples were collected at this time. All samples were compared to parallel cultures maintained in 20% FBS. In vertebral marrow cultures grown in the presence of 20% FBS, Northern blot analysis confirmed that IGF-I, IGFBP-3, and IGFBP-5 were upregulated by T_3 (10^{-8} M) (Fig. 4). IGFBP-2 and IGFBP-4 gene expression were also upregulated but to a lesser degree, while IGFBP-6 mRNA levels did not change in response to T_3 addition. In the serum-free control vertebral cultures, IGFBP-3 transcripts were not detected, while IGFBP-2, IGFBP-4, and IGFBP-5 transcripts were visible but their levels were drastically decreased. Only IGFBP-6 levels were unaffected by the removal of the serum. When T_3 (10^{-8} M) was added to the serum-free media, gene expression of several of the IGFBPs was upregulated. IGFBP-2 levels were upregulated eightfold, IGFBP-4 levels increased fourfold, and IGFBP-5 mRNA levels increased sixfold. IGFBP-6 levels did not change in response to T_3 addition in serum-free cultures. The IGF-I transcripts were not detected in the absence of serum, but the gene was strongly expressed when T_3 was added.

Following probing for the detection of mRNAs for the IGF components, the membranes were hybridized with gene probes that assess the osteoblast phenotype (Fig. 4). OC, Coll I, and BSP mRNAs were each abundant in vertebral marrow cultures maintained in medium containing 20% FBS and dex (10^{-7} M), ascorbic acid, and β-GP. The addition of T_3 (10^{-8} M) further increased OC steady-state mRNA levels (1.5-fold) and Coll I mRNA (1.6-fold), and only moderately increased BSP mRNA levels (7% increase). On the other hand, vertebral marrow cells *maintained in serum-free medium* supplemented with dex, ascorbic acid, and β-GP had undetectable OC, Coll I, and BSP gene expression, even when T_3 was present. Thus, the osteoblast phenotype was

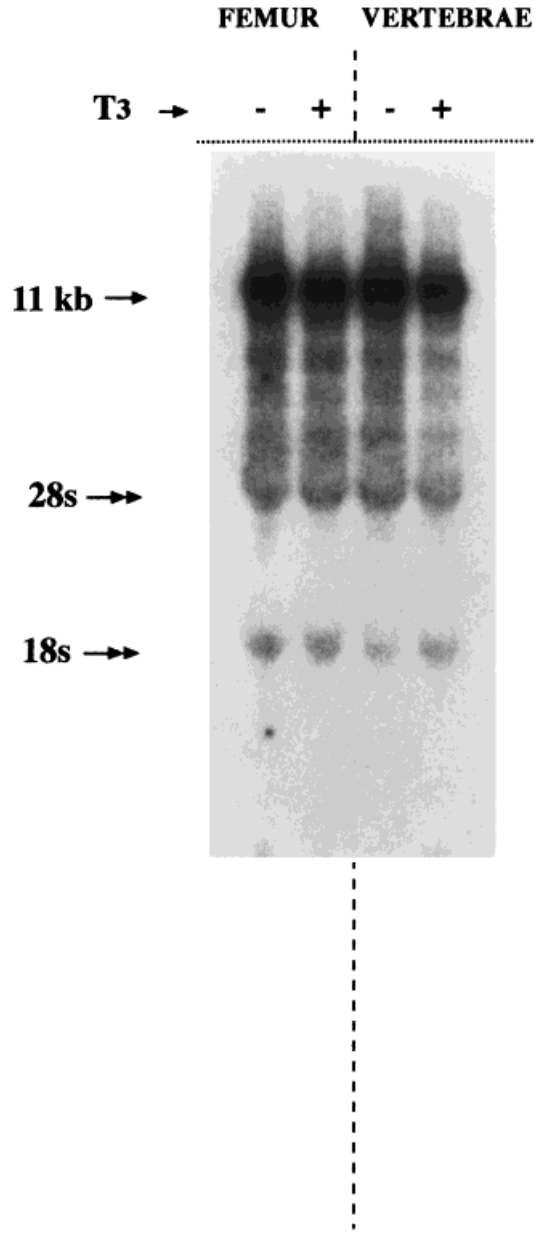

Fig. 3. IGF-I receptor mRNA expression in femoral and vertebral bone marrow stromal cell cultures. Total RNA from cells grown in the medium which supports osteoblast differentiation, with or without the addition of T_3 (10^{-8} M) was harvested on Day 6 of secondary cultures. 20 μg/lane was used for Northern analysis, with a cDNA probe specific for the rat IGF-I receptor and visualized after 100 h of film exposure.

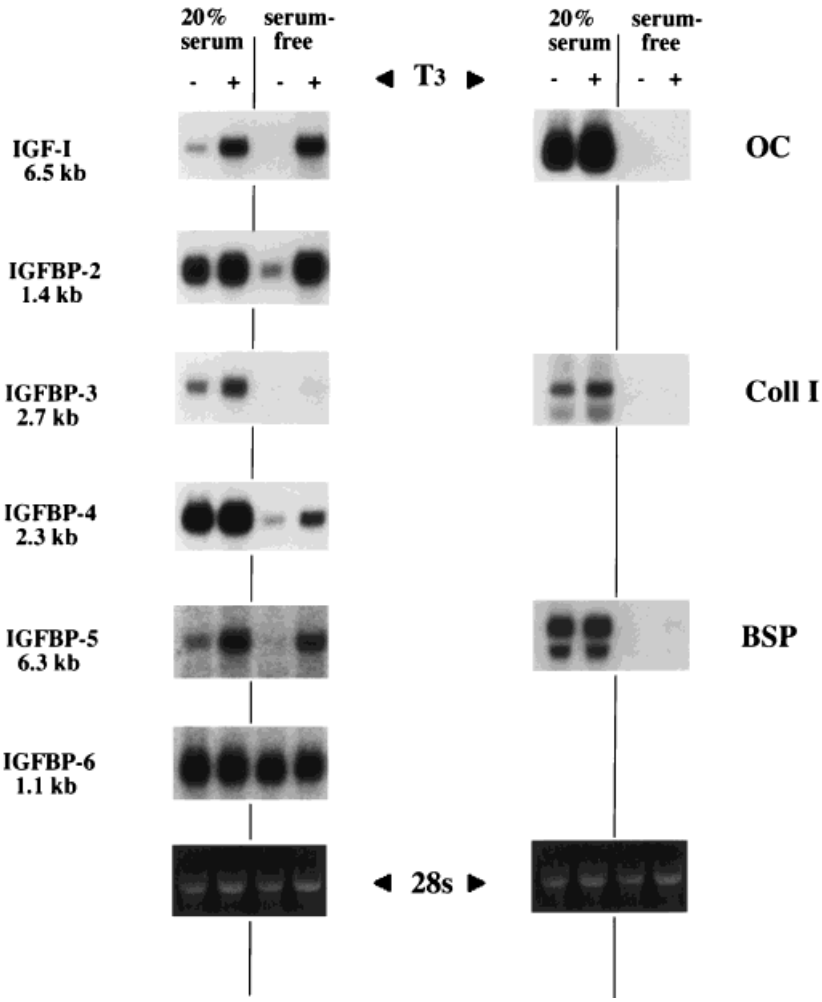

Fig. 4. Regulation of gene expression by T_3 (10^{-8} M) in the presence or absence of serum. Osteogenic vertebral marrow cell cultures were maintained in serum-containing (20% FBS) or serum-free medium, as described in Materials and Methods. Total RNA was extracted on Day 12 of culture. 5 μg/lane was used for Northern analysis, as described in Figure 1. Membranes were probed sequentially for the detection of IGFBP mRNAs, followed by probing for the expression of genes of the osteoblast phenotype. The lower panels show the ethidium bromide-stained 28s ribosomal band to confirm equal loading.

absent in serum-free marrow cultures, with or without the addition of T_3, implicating that the cells responsible for gene expression of the IGF components in vertebral cultures are *not* the differentiated osteoblasts.

Consistent with the observed increase in IGF-I mRNA by the addition of T_3 to serum-free vertebral marrow cultures, radioimmunoassay indicated that IGF-I protein in the serum-free conditioned medium increased to 1.8-fold of control level. Control levels of IGF-I secretion were notably higher in vertebral cultures maintained in 20% serum compared to serum-free cultures, and again the supplementation with T_3 increased IGF-I protein levels (Fig. 5). Western immunoblotting assays were used to identify IGFBP proteins in serum-free conditioned medium collected from vertebral cultures. Under the serum-free culture conditions used, we were unable to detect any IGFBP proteins in either control or T_3-treated vertebral marrow cultures (data not shown), even though IGF-I protein was detected in these same serum-free samples (Fig. 5). This emphasizes the dependence on serum supplementation for complete protein expression and for detailed hormone response studies in bone marrow osteogenic cell cultures.

Effects of Dex on IGFBP Transcript Levels

Previous studies in our laboratory revealed that dex supports the differentiation in second-

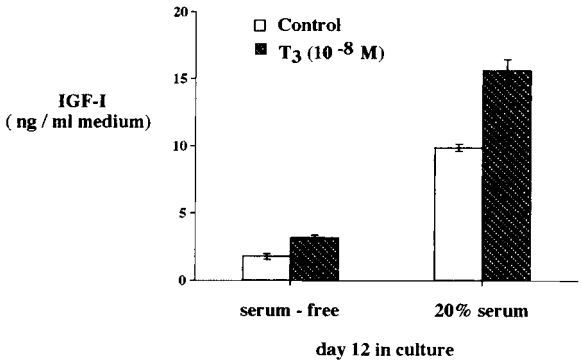

Fig. 5. Stimulation of IGF-I protein secretion by T_3 in vertebral marrow stromal cells grown in serum-free vs. serum-containing culture medium. During the last 4 days of treatment, cells were incubated in a serum-free medium containing $\pm T_3$ (10^{-8} M), or in medium containing 20% FBS $\pm T_3$ (10^{-8} M). Conditioned medium was collected and processed for IGF-I assay. Each bar represents the mean $\pm$ SEM of three replicates per treatment.

ary culture of both vertebral and femoral rat marrow stromal cells into osteoblasts. Dex supplementation (10^{-8} and 10^{-7} M) did not increase IGF-I steady-state mRNA levels in femoral marrow cultures, but did increase IGF-I gene expression in vertebral cultures. Femoral bone marrow cultures did not survive beyond Day 6 unless dex was present at 10^{-7} M [Milne et al., 1998b]. In the present study, we analyzed IGFBP mRNA levels in vertebral and femoral marrow cultures treated with dex. A representative Northern blot probed sequentially for each of the IGFBPs is shown in Figure 6. Vertebral marrow stromal cells under basal conditions (no dex supplementation) express transcripts encoding IGFBP-2, -3, -4, -5, or -6. Six days of dex treatment (either 10^{-8} or 10^{-7} M, which supports full expression of the osteoblast phenotype) did not appreciably change the vertebral steady-state levels of IGFBP-2, -3, -4, or -6. In contrast, IGFBP-5 transcripts were potently and dose-dependently decreased (up to 9-fold) by dex treatment in vertebral cultures. Femoral marrow stromal cells under basal conditions (no dex) expressed only IGFBP-4 and IGFBP-6. IGFBP-4 mRNA levels in femoral cultures were dose-dependently decreased (up to 8-fold) in the presence of dex. IGFBP-2, -3 and -5 transcripts were not detectable in femoral cells under basal conditions, nor after exposure to dex. Once again neither vertebral nor femoral cultures expressed IGFBP-1. This was true even in the absence of dex (data not shown).

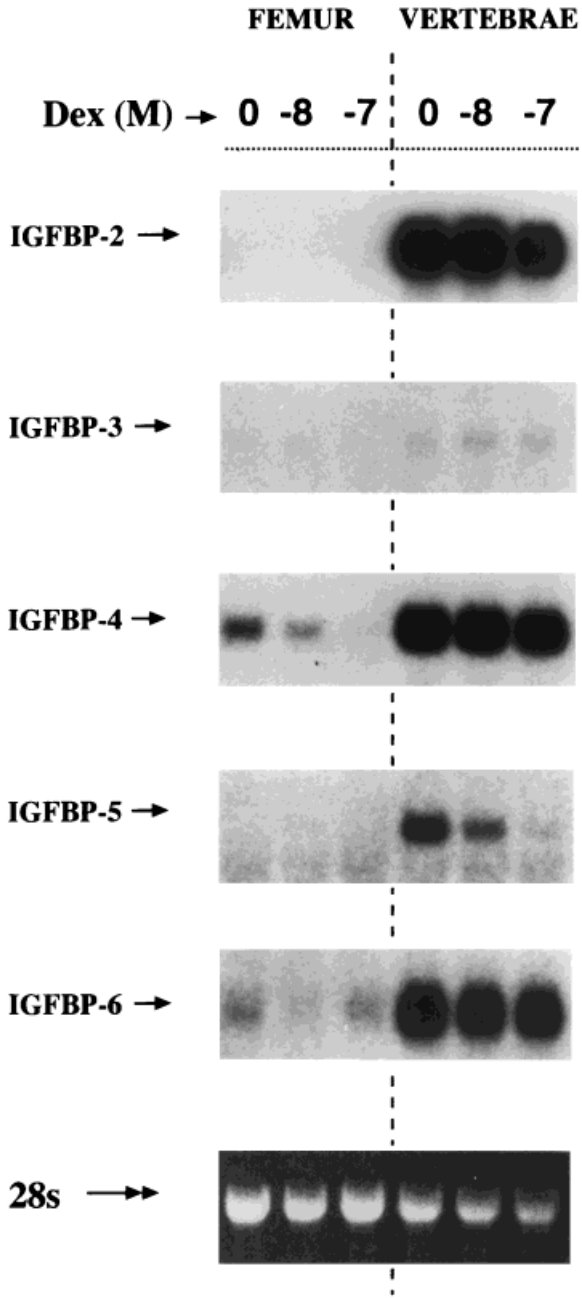

Fig. 6. Effects of dex on IGF binding protein gene expression in femoral vs. vertebral marrow cell cultures. Marrow cells were grown in primary culture in the presence of dex (10^{-8} M) and then passaged and maintained in secondary culture in vehicle (C), or 10^{-8} or 10^{-7} M dex. Total RNA was isolated on Day 6, and 5 μg/lane was used for Northern blot analysis. The membrane was probed sequentially for each of the IGFBP mRNAs. The lower panel shows the ethidium bromide stained 28s ribosomal bands.

Importantly, vertebral marrow cultures had much higher IGFBP mRNA levels than femoral cultures for all the detected IGFBP transcripts. Previously, we found that with these culture

conditions, the presence of dex (10^{-7} M in secondary culture) supports OC gene expression and alkaline phosphatase enzyme activity for both femoral and vertebral marrow cells [Milne et al., 1998b]. Thus, even with conditions where the osteoblast phenotype is maintained, femoral cultures have very low levels of expression of the IGF regulatory components while these transcripts were abundant in vertebral cultures. This again suggests that the cells expressing the IGF component genes in vertebral marrow stromal cell cultures are not differentiated osteoblasts and demonstrate a difference in the marrow stromal cell populations between the two skeletal sites.

DISCUSSION

The anabolic effects of IGFs on bone are well documented. IGF action is modulated by IGF binding proteins, which are present in the circulation and are also produced locally by bone cells [Conover, 1996]. In the adult, hormonal control of bone formation and resorption is mediated by cells of the osteoblast lineage, including marrow stromal cells [Rodan, 1998]. It has been reported that osteoblasts synthesize IGF-I, -II, and IGFBPs, and gene expression is under the hormone control of numerous local and systemic factors [Schmid, 1995; Conover, 1996]. Marrow stromal cells also produce IGF-I, -II, and IGFBPs [Zhang et al., 1991; Schmid, 1995; Malpe et al., 1997; Rodan, 1998] and it is likely that stromal cells contribute substantially to the amount of IGFs and IGFBPs deposited in the skeletal matrix [Rosen et al., 1994]. It has been suggested that variations in the levels of expression of the IGF system components play a role in the differential regulation of bone metabolism at separate skeletal sites [Malpe et al., 1997]. In the present study, we compared IGFBP gene expression in bone marrow stromal cell cultures derived from young adult rat femurs and vertebrae. We also studied hormone modulation of the IGFBPs by investigating the effects of T_3 and dex in femur- and vertebrae-derived bone marrow cell cultures.

Our observations indicate that distinct differences exist in the expression of the IGF regulatory components between bone marrow cells obtained from different skeletal sites yet cultured under identical ex vivo conditions. Also, our studies indicate that T_3 supplementation in serum-free vertebral cultures upregulates steady-state transcript levels of IGF-I and IGFBP-2, -3, -4, and -5. This is in agreement with earlier studies on the T_3 regulation of IGFBP expression by osteoblasts [Schmid et al., 1992; Glantschnig et al., 1996]. Using osteoblast-like cells from neonatal rat calvariae, Schmid et al. [1992] reported that the addition of T_3 to serum-free cultures increased the net production of IGFBP-2 and -3 proteins. In the mouse osteoblastic cell line MC3T3-E1, IGFBP-4 mRNA was also upregulated in the presence of T_3 [Glantschnig et al., 1996]. Taken together, these findings predict that the thyroid hormone has an important regulatory role in IGFBP production by osteoblasts and marrow stromal cells in vivo. However, when our bone marrow stromal cell cultures were exposed to the serum-free conditions necessary for assaying IGFBPs, we found that the gene markers typical of differentiated osteoblasts, e.g., osteocalcin, Coll I, BSP, ceased to be expressed. Thus, interpretation of bone cell culture data under serum-free conditions must be brought into perspective with regard to osteoblast metabolism. Based upon results presented here, we hypothesize that the marrow stromal cells in close proximity to vertebral osteoblasts and osteoprogenitors secrete factors, including IGF-I and IGFBPs, which modulate osteoblast differentiation and activity in vivo, and that T_3 is acting through these marrow stromal cells to hormonally regulate osteoblast activity in vertebrae.

It is now acknowledged that bone cells differ depending on their site of origin [Kasperk et al., 1995] and respond differently to circulating hormones [Heersche et al., 1993; Bland, 2000]. For example, the thyroid hormone is necessary for normal skeletal growth and maintenance [Stern, 1996], but elevated thyroid hormone concentrations, such as those found in hyperthyroidism, decrease bone mass at the hip more than the spine [Diamond et al., 1990; Ongphiphadhanakul et al., 1992; Suwanwalaikorn et al., 1996; Gouveia et al., 1997]. IGF-I is complexed to the IGFBPs, which serve to protect the half-life of IGF-I in vivo or to inhibit its action on target cells. Additionally, specific IGFBPs function to potentiate the action of IGFs and have also been reported to alter cellular functions even in the absence of IGF-I and IGF-II [Rajaram et al., 1997; Rechler 1997]. Thus, in order to better understand the regulatory actions of IGFs on the adult skele-

ton, it is essential to characterize the expression of the IGFBPs in bone cells at different anatomic sites. IGFBP expression has been studied extensively in numerous cell lines and in fetal and neonatal calvarial osteoblast cultures [Hassager et al., 1992; McCarthy et al., 1994; Chen et al., 1998], and more recently in adult human bone cell cultures [Malpe et al., 1997; Cheng et al., 1998]. Cheng et al. analyzed IGFBPs in marrow stromal cell cultures derived from human rib bones and detected IGFBP-2 through -6 [Cheng et al., 1998]. Malpe et al. [1997] also measured IGFBP levels (-3,-4, and -5) and noted site-specific differences in human bone cells derived from five separate skeletal sites: calvaria, mandible, rib, vertebra, and vertebral bone marrow.

However, all of these bone cell sites studied [Malpe et al., 1997; Cheng et al., 1998] are part of the axial skeleton, whereas vertebrae vs. femur-derived marrow cultures enable a comparative investigation of the axial and appendicular skeleton. Different parts of the skeleton serve different functions in the adult. At skeletal maturity, the majority of the marrow of the axial skeleton is active in hematopoiesis, whereas, in the long bones of the appendicular skeleton, the majority of the marrow space is occupied by adipocytes, with hematopoietically active marrow confined to the metaphyses and epiphyses [Bianco and Riminucci, 1998]. There is convincing evidence that the marrow hematopoietic tissue modulates bone metabolism [Shinar and Rodan, 1993]. Thyroid hormone stimulates IGF-I production in bone and IGF-I also enhances hematopoiesis [Dainiak et al., 1986; Merchav et al., 1988, 1992; Sainteny et al., 1990; Merchav, 1998]. The differing effects of systemic hormones on bone at separate skeletal sites may in part be a consequence of the manner in which the neighboring marrow cells respond to these hormones.

Our characterization of IGFBPs and the effects of dex on their gene expression emphasize the differences between axial and appendicular marrow cell populations. The data with IGFBP-5 expression by vertebral cultures confirm the work of other investigators with human rib marrow stromal cultures [Cheng et al., 1998], showing that IGFBP-5 mRNA levels are dramatically decreased by dex. Characterization of the expression of IGF-II and the proteases that cleave the IGFBPs [Clemmons, 1997] is necessary for us to entirely describe the IGF regulatory axis on femur- vs. vertebrae-derived cultures. We have found that while dex supports osteoblast differentiation from bone marrow progenitor cells present in both femurs and vertebrae [Milne et al., 1998b], dex does not support substantial expression of the majority of the IGF regulatory components in femoral marrow cultures.

In conclusion, femur and vertebrae-derived marrow stromal cells in culture differentially express IGF-I and the IGFBPs. T_3 supplementation alters the expression of IGFBPs in vertebral cultures and causes a net increase in IGF-I protein. Dex supplementation also alters the level of expression of IGFBP genes in both marrow cell cultures. These findings suggest that this heterogeneity of bone cell responses at different sites in the skeleton is due in part to the modulation of expression of genes within the IGF regulatory system. The findings also lead to new questions: are the osteoblasts themselves inherently different at the two skeletal sites or is there a difference in the composition of the surrounding marrow stromal cell population that controls the function of different parts of the skeleton? Our data suggest that the answer lies within the complex interactions of the various cell types that make up the total bone marrow system.

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