Journal of Cellular Biochemistry 66:77–86 (1997)

Growth Factor Regulation of Insulin-Like Growth Factor Binding Protein-6 Expression in Osteoblasts

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Abstract Previously we have shown that transforming growth factor β (TGF β) 1, basic fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) BB inhibit the synthesis of insulin-like growth factor (IGF) II, but their effects on IGF binding protein (IGFBP)-6 in osteoblast cultures are not known. IGFBP-6 binds IGF II with high affinity and prevents IGF II–mediated effects, so that a possible mode of regulating the IGF II available to bone cells would be by changing the levels of IGFBP-6. To enhance our understanding of the actions of growth factors on the IGF II axis in bone, we tested the effects of TGF β1, basic FGF, PDGF BB, IGF I, and IGF II on the expression of IGFBP-6 in cultures of osteoblast-enriched cells from 22 day fetal rat calvariae (Ob cells). Treatment of Ob cells with TGF β1 caused a time- and dose-dependent decrease in IGFBP-6 mRNA levels, as determined by Northern blot analysis. The effect was maximal after 48 h and observed with TGF β1 concentrations of 0.04 nM and higher. TGF β1 also decreased IGFBP-6 polypeptide levels in the medium, as determined by Western immunoblot analysis. Cycloheximide at 3.6 μM decreased IGFBP-6 transcripts and prevented the effect of TGF β1. The decay of IGFBP-6 mRNA in transcriptionally arrested Ob cells was not modified by TGF β1. In addition, TGF β1 decreased the rates of IGFBP-6 transcription as determined by a nuclear run-on assay. In contrast, basic FGF, PDGF BB, IGF I, and IGF II did not change IGFBP-6 mRNA levels in Ob cells. In conclusion, TGF β1 inhibits IGFBP-6 expression in Ob cells by transcriptional mechanisms. Since IGFBP-6 binds IGF II and prevents its effects on bone cells, decreased synthesis of IGFBP-6 induced by TGF β1 could be a local feedback mechanism to increase the amount of IGF II available in the bone microenvironment. J. Cell. Biochem. 66:77–86, 1997.

Key words: skeletal cells; transforming growth factor β; transcripts; bone formation; local factors

Insulin-like growth factor (IGF) II is one of the most prevalent growth factors synthesized by skeletal cells [Frolik et al., 1988; Mohan et al., 1988]. IGF II, like IGF I, has modest mitogenic activity for cells of the osteoblastic lineage and increases the differentiated function of the osteoblast [McCarthy et al., 1989]. IGF II increases type I collagen gene expression and inhibits the synthesis of interstitial collagenase, and through these two mechanisms it plays a role in the maintenance of the bone matrix [McCarthy et al., 1989; Canalis et al., 1995]. The levels and activity of IGF II in bone can be regulated by changes in its synthesis, receptor binding, and IGF binding proteins (IGFBP) [Delany et al., 1994]. Although systemic hormones modify the synthesis of IGF I by skeletal cells, they do not regulate the synthesis of IGF II [Delany et al., 1994; Canalis et al., 1991]. In contrast, other growth factors secreted by skeletal cells, such as transforming growth factor β (TGF β) 1, basic fibroblast growth factor (FGF), and platelet derived growth factor (PDGF) BB inhibit the expression of both IGF I and IGF II by the osteoblast [Canalis et al., 1993; Gabbitas et al., 1994]. Among these growth factors, TGF β1 is the most potent inhibitor of IGF II synthesis in osteoblast cultures.

There are six known IGFBPs, and, although they are all synthesized by osteoblasts, their level of expression varies with the cell line studied and culture conditions used [Rechler, 1993; Hassager et al., 1992; McCarthy et al.,

Contract grant sponsor: National Institute of Diabetes and Digestive and Kidney Diseases, contract grant number DK 42424.

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Received 23 January 1997; accepted 6 March 1997

1994; Okazaki et al., 1994]. Although the function of all the IGFBPs in skeletal tissue is not completely understood, IGFBP-6 has been shown to have unique properties. IGFBP-6 binds IGF II with 20–100 times higher affinity than IGF I and selectively blocks IGF II effects on myoblasts and osteoblasts [Bach et al., 1993, 1994; Kiefer et al., 1992]. These studies suggest that changes in IGFBP-6 have the potential to modify the activity and available levels of IGF II in the musculoskeletal system. An increase or decrease in IGFBP-6 levels by the osteoblast could result in a converse change in available IGF II in the bone microenvironment.

Studies on the regulation of IGFBP-6 synthesis have been limited. Retinoic acid is a major inducer of IGFBP-6 expression in both skeletal and nonskeletal cells, and the IGFBP-6 promoter contains retinoic acid responsive elements [Martin et al., 1994; Gabbitas and Canalis, 1996a; Sheikh et al., 1993; Zhou et al., 1996; Zhu et al., 1993]. Glucocorticoids also induce the expression of IGFBP-6 in cultured osteoblasts, while other steroid hormones, such as estrogens, inhibit ovarian expression of the binding protein in vivo [Gabbitas and Canalis, 1996b; Rohan et al., 1993]. However, there is little information about the regulation of IGFBP-6 by growth factors, particularly in skeletal cells.

TGF β, basic FGF, and PDGF BB have complex effects on bone formation and resorption. Some of these are probably due to direct actions on specific genes expressed by the osteoblast, whereas others may be indirect and mediated by effects on the IGF/IGFBP axis [Canalis et al., 1988, 1993; Gabbitas et al., 1994; Centrella et al., 1987; Canalis et al., 1988; Hock and Canalis, 1994]. Although TGF β1, basic FGF, and PDGF BB inhibit IGF I and IGF II synthesis in osteoblasts, not all of their actions, particularly in the case of TGF β1, are opposite to those of IGFs. This suggests the possibility of additional direct effects of the growth factors on the osteoblast or the existence of local feedback mechanisms. We postulated that a possible feedback mechanism could be via the regulation of IGFBP-6, which could result in changes in the levels of IGF II available to bone cells.

The present studies were undertaken to examine the effects of TGF β1, basic FGF, PDGF BB, and IGFs themselves on IGFBP-6 synthesis in cultures of osteoblast enriched cells from 22 day fetal rat calvariae (Ob cells) and to determine possible mechanisms of action involved.

MATERIALS AND METHODS

Culture Technique

The culture method used was described in detail previously [McCarthy et al., 1988]. Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were sacrificed by blunt trauma to the nuchal area. This project was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of the parietal bone using bacterial collagenase (CLS II; Worthington Biochemical, Freehold, NJ). Cell populations harvested from the third to the fifth digestions were cultured as a pool and were previously shown to have osteoblastic characteristics [McCarthy et al., 1988]. Ob cells were plated at a density of 8,000–12,000 cells/cm^2 and cultured in a humidified 5% CO_2 incubator at 37°C until reaching confluence (about 50,000 cells/cm^2). For the nuclear run-on experiment, first passage cultures were used and subconfluent primary cells were trypsinized, replated, and grown to confluence. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from Summit Biotechnologies, Fort Collins, CO). At confluence, the cells were rinsed and transferred to serum-free medium for 16–24 h, when they were again rinsed with serum-free medium and exposed to test or control medium in the absence of serum for 2–48 h. In 48 h treated cultures, the medium was replaced after 24 h with fresh control or test solutions. Basic FGF, PDGF BB (both from Austral, San Ramon, CA), and cycloheximide (Sigma Chemical Co., St. Louis, MO) were added directly to the medium. Recombinant human TGF β1 (a gift from Genentech, South San Francisco, CA) was dissolved in 5 mM HCl and diluted 1:3,000 or greater in DMEM, recombinant human IGF I (Austral) was dissolved in 20 mM citrate and diluted 1:1,000 in DMEM, and recombinant human IGF II (a gift from Eli Lilly Research Laboratories, Indianapolis, IN) was dissolved in 100 mM acetic acid and diluted 1:100 in DMEM. 5,6-dichlorobenzimidazole riboside (DRB) (Sigma) was dissolved in absolute ethanol and diluted 1:200 in DMEM. All experimental groups were exposed to an equal concentration of solvent. For RNA analysis, the cell layer was extracted with guanidine thiocyanate at the end of the incubation and stored at −70°C.

For the nuclear run-on assay, nuclei were isolated by Dounce homogenization. For protein levels, the medium was aspirated and stored at −70°C in the presence of 0.1% Tween 20 (Sigma).

Northern Blot Analysis

Total cellular RNA was isolated with guanidine thiocyanate, at acid pH, followed by phenol-chloroform (Sigma) extraction and ethanol precipitation or by RNeasy kit per the manufacturer's instructions (Qiagen, Chatsworth, CA) [Chomczynski and Sacchi, 1987]. The RNA recovered was quantitated by spectrometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel following denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, demonstrating RNA loading of the various experimental samples. The RNA was then blotted onto GeneScreen Plus charged nylon (DuPont, Wilmington, DE) and uniformity of the transfer documented by revisualization of ribosomal RNA. A 246 base pair (bp) Pst I restriction fragment of the rat IGFBP-6 cDNA (kindly provided by Dr. S. Shimasaki, La Jolla, CA) was purified by agarose gel electrophoresis [Zhu et al., 1993; Shimasaki et al., 1991]. IGFBP-6 cDNA was labeled with [α-^{32}P]-deoxycytidine triphosphate and [α-^{32}P]-deoxyadenosine triphosphate (50 µCi each at a specific activity of 3,000 Ci/mmol) (DuPont) using the random hexanucleotide primed second strand synthesis method [Feinberg and Vogelstein, 1984]. Hybridizations were carried out at 42°C for 16–72 h, and posthybridization washes were performed at 65°C in 0.5× saline-sodium citrate (SSC). The blots were stripped and rehybridized with a 752 bp BamH I–Sph I restriction fragment of the murine 18S ribosomal RNA cDNA (American Type Culture Collection, Rockville, MD) under the same conditions. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak, Rochester, NY) employing Cronex Lightning Plus intensifying screens (DuPont). Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of three or more cultures.

Nuclear Run-On Assay

To examine changes in the rate of transcription, we isolated nuclei by Dounce homogenization in a Tris buffer containing 0.5% Nonidet P-40. Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 µM each adenosine, cytidine, and guanosine triphosphates, 150 units RNasin (Promega, Madison, WI), and 250 µCi [α-^{32}P]-uridine triphosphate (3,000 Ci/mM) (DuPont) [Greenberg and Ziff, 1984]. RNA was isolated by treatment with DNase I and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized plasmid pBluescript SK+ DNA containing about 1 µg of the IGFBP-6 cDNA was immobilized onto GeneScreen Plus by slot blotting according to the manufacturer's directions (DuPont). The plasmid vector pGL2-Basic (Promega) was used as a control for nonspecific hybridization, and murine 18S cDNA was used to confirm uniformity of the radioactive counts applied to each membrane. Equal counts per minute of [α-^{32}P]-RNA from each sample were hybridized to cDNAs at 42°C for 72 h and washed in 0.1× SSC at 65°C for 30 min. Hybridized cDNAs were visualized by autoradiography.

Western Blot Analysis

Medium aliquots were mixed with Laemmli sample buffer to give a final concentration of 2% sodium dodecyl sulfate and fractionated by polyacrylamide gel electrophoresis on a 15% denaturing gel in the absence of reducing agents [Laemmli, 1970]. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) blocked with 2% bovine serum albumin and exposed to a 1:3,000 dilution of rabbit antiserum raised against rat IGFBP-6 (kindly provided by S. Shimasaki) in 1% bovine serum albumin overnight [Liu et al., 1993]. Blots were exposed to goat antirabbit IgG antiserum conjugated to horseradish peroxidase, washed, and developed with a horseradish peroxidase chemiluminescent detection reagent (DuPont). IGFBP-6 was identified by migration analogous to recombinant human IGFBP-6 (Austral), which was visualized with rabbit antihuman IGFBP-6 antiserum (Austral). Western immunoblots are representative of three or more cultures.

Statistical Methods

Values are expressed as means ± SEM. Data on mRNA decay were plotted by linear regression, and the slopes of the regression lines obtained for control and treated cells were ana-

lyzed for significant differences using the method of Sokal and Rohlf [1981].

RESULTS

Northern blot analysis of total RNA from Ob cells revealed a predominant IGFBP-6 transcript of 1.3 kb (Fig. 1). Continuous treatment of Ob cells with TGF β1 caused a time-dependent decrease in IGFBP-6 steady-state mRNA levels. After exposure to TGF β at 0.4 nM, the effect was initially detected after 24 with a 58 ± 3% (n = 8) decrease in IGFBP-6 mRNA levels as determined by densitometry, and, after 48 h, TGF β1 inhibited IGFBP-6 transcripts by 76 ± 3% (n = 8) (Fig. 1). The effect of TGF β1 was dose-dependent as well, and continuous treatment of Ob cells with TGF β1 for 24 or 48 h at 0.04–1.2 nM decreased IGFBP-6 transcripts by as much as 90% (Fig. 2). Western immunoblot analysis of the medium from untreated Ob cells demonstrated the presence of immunoreactive IGFBP-6 (Fig. 3). The antirat IGFBP-6 antibody did not cross-react with a recombinant human IGFBP-6 standard, but, when a single blot was divided and part exposed to antibody against the rat protein and part to the antibody against the human protein, comigration of rat and human IGFBP-6 was demonstrated (Fig. 3). TGF β1 at 0.4 nM for 24 h decreased the levels of immunoreactive IGFBP-6 in the culture medium by 41 ± 7% (n = 4).

To determine whether or not the effects observed on IGFBP-6 mRNA levels were dependent on protein synthesis, we treated Ob cells with TGF β1 in the presence or absence of cycloheximide at 3.6 μM. In earlier experiments, cycloheximide at doses of 2 μM and higher was found to inhibit protein synthesis in Ob cell cultures by 80–85% [Centrella et al., 1991]. Northern blot analysis revealed that treatment with cycloheximide for 24 h caused a modest decrease in IGFBP-6 mRNA levels and prevented the inhibitory effect of TGF β1 (Fig. 4).

To examine whether or not the effect of TGF β1 on IGFBP-6 mRNA levels was due to changes in transcript stability, cultures of Ob cells were exposed to DMEM or TGF β1 for 60 min and then treated with the RNA polymerase II inhibitor DRB in the absence or presence of TGF β1 at 0.4 nM for 6, 16, or 24 h [Zandomeni et al., 1983]. About 75% of Ob cells are viable in the presence of DRB for 24 h, but cell viability is impaired following exposure to DRB for longer periods of time, as determined by trypan blue exclusion [E. Canalis, unpublished observations]. After 24 h of DRB exposure, a 25% decay in IGFBP-6 mRNA was detected, and the change was not significantly different in control and TGF β1–treated samples (Fig. 5). The half-life of IGFBP-6 mRNA in transcriptionally arrested Ob cells was estimated to be more than

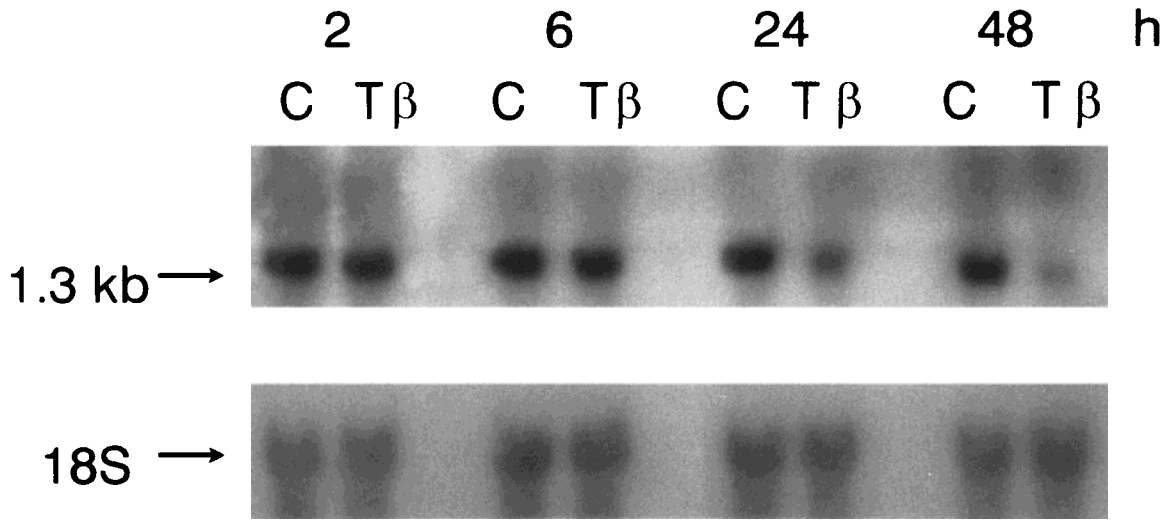

Fig. 1. Effect of transforming growth factor β1 (Tβ) at 0.4 nM on IGFBP-6 mRNA expression in cultures of Ob cells treated for 2, 6, 24, or 48 h. Total RNA from control (C) or TGF β–treated cultures was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled rat IGFBP-6 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-6 mRNA was visualized by autoradiography and is shown in the **upper panel,** while 18S ribosomal RNA is shown in the **lower panel.**

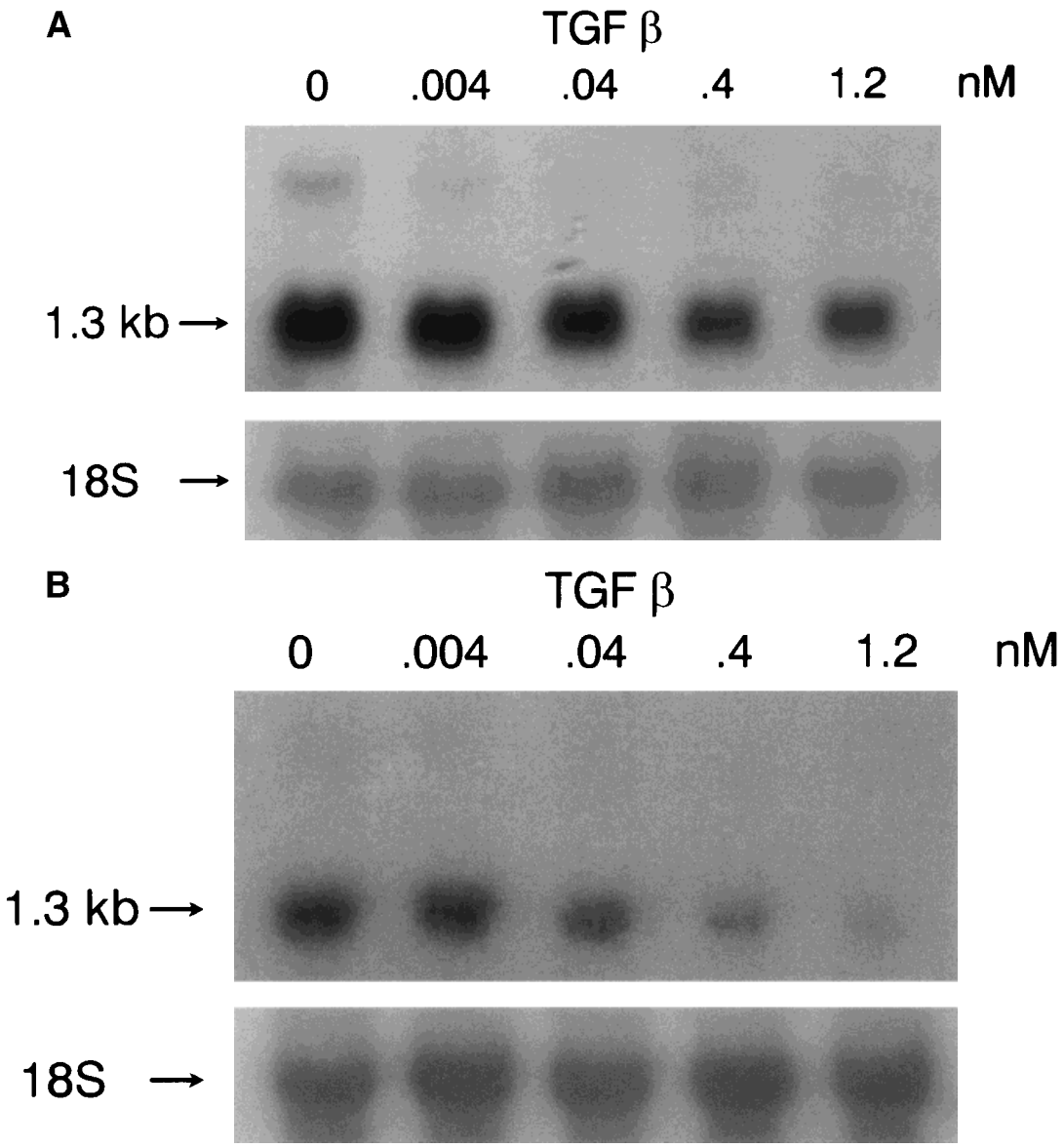

Fig. 2. Effect of transforming growth factor β1 (TGF β) at 0.004–1.2 nM on IGFBP-6 mRNA expression in cultures of Ob cells treated for **(A)** 24 h and **(B)** 48 h. Total RNA from control (0) or TGF β–treated cultures was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled rat IGFBP-6 cDNA. The blots were stripped and rehybridized with labeled murine 18S cDNA. IGFBP-6 mRNA was visualized by autoradiography and is shown in the **upper panels,** while 18S ribosomal RNA is shown in the **lower panels.**

48 h by extrapolation of the values obtained in the first 24 h. To confirm whether or not TGF β1 modified the transcription of the IGFBP-6 gene, a nuclear run-on assay was performed on nuclei from Ob cells treated for 6 h. This assay demonstrated that TGF β1 decreased the rate of IGFBP-6 transcription by 50% (Fig. 6).

In contrast to TGF β1, treatment with either basic FGF at 6 nM, PDGF BB at 3.3 nM, or IGF I or II at 100 nM for 2–48 h did not change IGFBP-6 mRNA levels in Ob cells (Figs. 7, 8).

DISCUSSION

Recently, we have shown that TGF β1, basic FGF, and PDGF BB act on the IGF/IGFBP axis in osteoblasts and decrease the expression of IGF I and IGF II in these cells [Canalis et al., 1993; Gabbitas et al., 1994]. The present investigation was undertaken to determine whether or not these three growth factors and IGFs themselves regulated IGFBP-6 expression in calvarial derived rat osteoblasts and to deter-

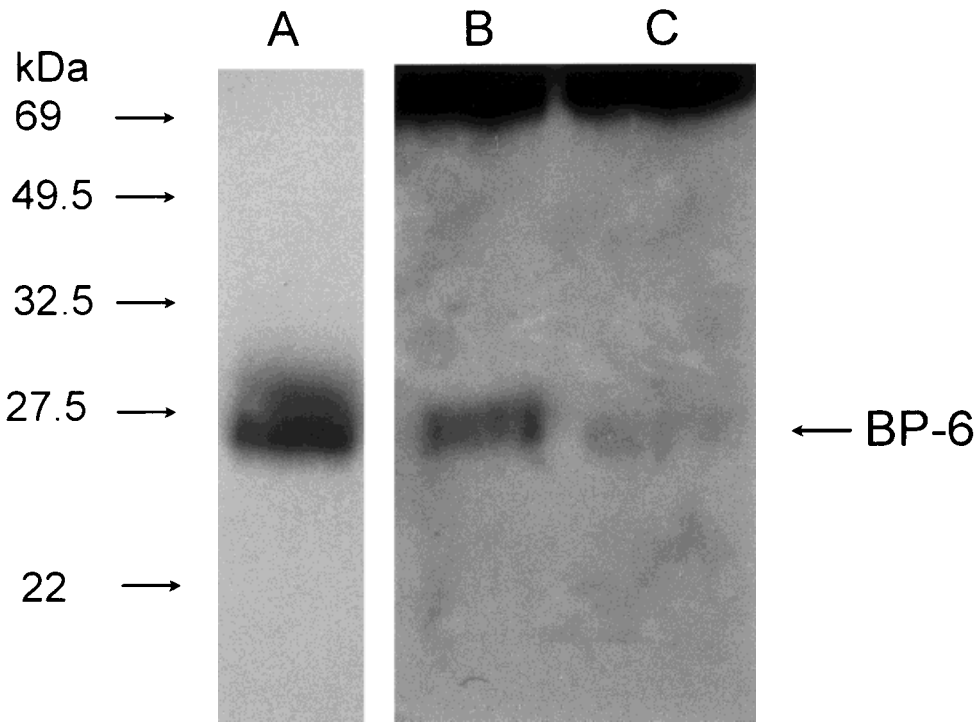

Fig. 3. Effect of transforming growth factor β1 (TGF β) on IGFBP-6 polypeptide levels in cultures of Ob cells treated for 24 h. **Lane A:** Western immunoblot of recombinant human IGFBP-6 standard detected using antihuman IGFBP-6 antiserum. **Lanes B, C:** Western immunoblot of conditioned medium from control cultures (B) or cultures treated with TGF β (C). Antirat IGFBP-6 antibody and a chemiluminescent second antibody detection system were utilized. Numbers in the left margin indicate the migration of molecular weight markers in kilodaltons (kDa).

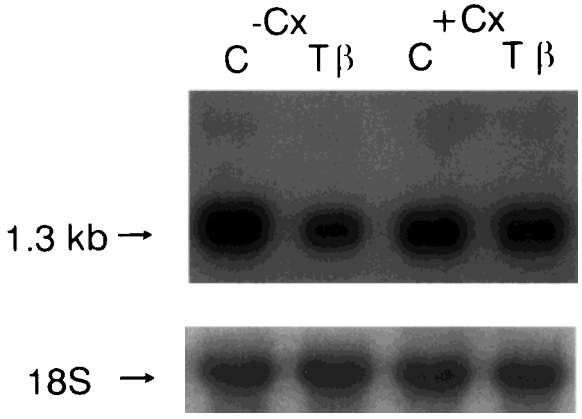

Fig. 4. Effect of transforming growth factor β1 (Tβ) in the presence (+) or absence (−) of cycloheximide (Cx) at 3.6 μM on IGFBP-6 mRNA expression in cultures of Ob cells treated for 24 h. Total RNA from control (C) or TGF β–treated cultures was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled rat IGFBP-6 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-6 mRNA was visualized by autoradiography and is shown in the **upper panel,** while 18S ribosomal RNA is shown in the **lower panel.**

mine the mechanisms involved. We demonstrated that TGF β1 decreases IGFBP-6 mRNA levels in Ob cells in a time- and dose-dependent manner and that the effect requires de novo protein synthesis. Experiments in transcriptionally blocked Ob cells, using the RNA polymerase II inhibitor DRB, revealed that TGF β1 did not modify IGFBP-6 mRNA decay during a 24 h period [Zandomeni et al., 1983]. This, in conjunction with a decrease in the rates of transcription, indicates that TGF β1 inhibits IGFBP-6 expression at the transcriptional level. However, because the half-life of IGFBP-6 mRNA in the presence of DRB was estimated at more than 48 h and TGF β1 inhibited IGFBP-6 transcripts by about 50% at 24 h in the absence of DRB, it is possible to infer that destabilization of mRNA might have occurred. Alternatively, the transcription inhibitor itself may have prolonged the half-life of IGFBP-6 mRNA in the blocking experiment, since such agents have been shown to inhibit the cellular pathways leading to mRNA degradation [Shyu et al., 1989]. TGF β1 also decreased IGFBP-6 polypeptide levels in the medium. The effect observed

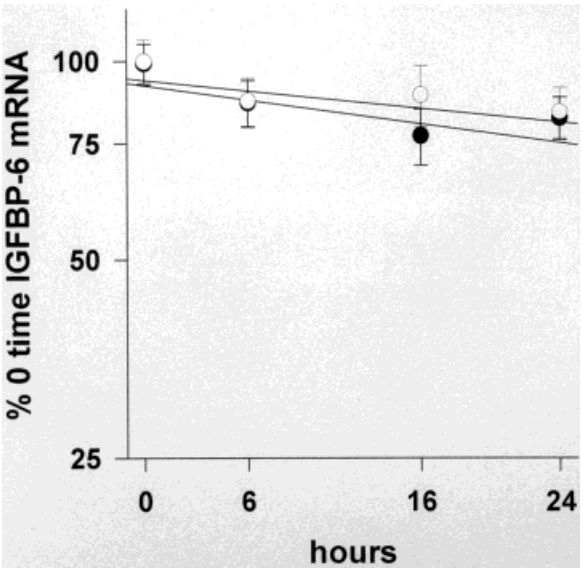

Fig. 5. Effect of transforming growth factor β1 (TGF β) on IGFBP-6 mRNA decay in transcriptionally arrested Ob cells. Cultures were treated with TGF β 60 min before and 6, 16, or 24 h after the addition of 5,6-dichlorobenzimidazole riboside (DRB). RNA was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled rat IGFBP-6 cDNA, visualized by autoradiography, and quantitated by densitometry. Ethidium bromide staining of ribosomal RNA was used to check uniform loading of the gels and transfer. Values are means ± SEM for three cultures. Values were obtained by densitometric scanning and are presented as percentage of IGFBP-6 mRNA levels relative to the time of DRB addition. Slopes were analyzed by the method of Sokal and Rohlf [1981] and not found to be statistically different.

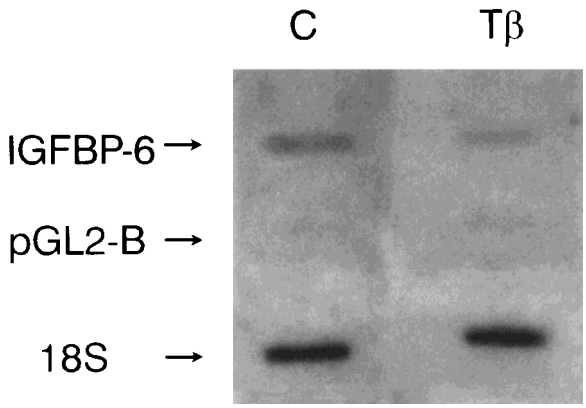

Fig. 6. Effect of transforming growth factor β1 (TGF β) on IGFBP-6 transcription rates in cultures of Ob cells treated for 6 h. Nascent transcripts from control (C) or TGF β (Tβ)–treated cultures were labeled in vitro with [α-^{32}P]-uridine triphosphate, and the labeled RNA was hybridized to immobilized cDNA for IGFBP-6. Murine 18S cDNA was used to demonstrate loading, and pGL2-Basic vector DNA was used as a control for nonspecific hybridization.

was unique to TGF β1 since other growth factors known to inhibit IGF I and IGF II synthesis, such as basic FGF and PDGF BB and IGFs themselves, did not modify IGFBP-6 mRNA levels. Other investigators have reported that TGF β1 causes an inhibition in IGFBP-6 levels in human fibroblasts [Martin et al., 1994]. However, the effect was primarily apparent in transformed cells, and TGF β1 had only a modest effect in normal fibroblasts. The mechanisms involved were not defined, and they may or may not involve regulation at the transcriptional level, as is demonstrated here in osteoblasts. TGF β inhibits the rat stromelysin gene through the TGF β inhibitory element (TIE), and, although the IGFBP-6 promoter contains a similar sequence, it shows some variations from the reported TIE sequence, and it is not known if it is functional and responsible for the effects observed [Matrisian et al., 1992].

Although the exact amount of TGF β1 available to skeletal cells is not known, its effects on IGFBP-6 synthesis were observed at doses that modify other parameters of osteoblastic cell function, suggesting that the inhibition of IGFBP-6 synthesis by TGF β1 is physiologically relevant [Centrella et al., 1987]. IGF II is abundant in skeletal tissue, and studies on its direct actions in bone cells and studies in mice, either with targeted IGF II gene disruption or overexpression of IGF II, have documented its relevance to skeletal growth [DeChiara et al., 1990; Ward et al., 1994]. Since IGFBP-6 binds IGF II and modulates its effects in osteoblasts, it is probable that the inhibition of IGFBP-6 by TGF β1 results in an increase in bioavailable IGF II in the bone microenvironment. Thus, the inhibition of IGFBP-6 by TGF β1 may act as a local feedback mechanism to maintain adequate levels of IGF in bone tissue.

TGF β has complex effects on bone remodeling and has a major impact on the IGF/IGFBP axis, suggesting that some of its long-term effects in bone could be mediated by changes in the synthesis or activity of IGF I, IGF II, or their binding proteins. In rat osteoblasts, TGF β1 decreases the synthesis of IGF I, IGF II, IGFBP-5, and IGFBP-6, although its actions on the expression of other IGFBPs in bone are not known [Canalis et al., 1993; Gabbitas et al., 1994; Canalis and Gabbitas, 1995]. Since IGFBP-5 enhances the effects of IGF I on bone cell growth, its inhibition by TGF β1 may contribute to a depression of the IGF/IGFBP axis in bone [Canalis and Gabbitas, 1995; Andress and Birnbaum, 1992]. In contrast, the inhibi-

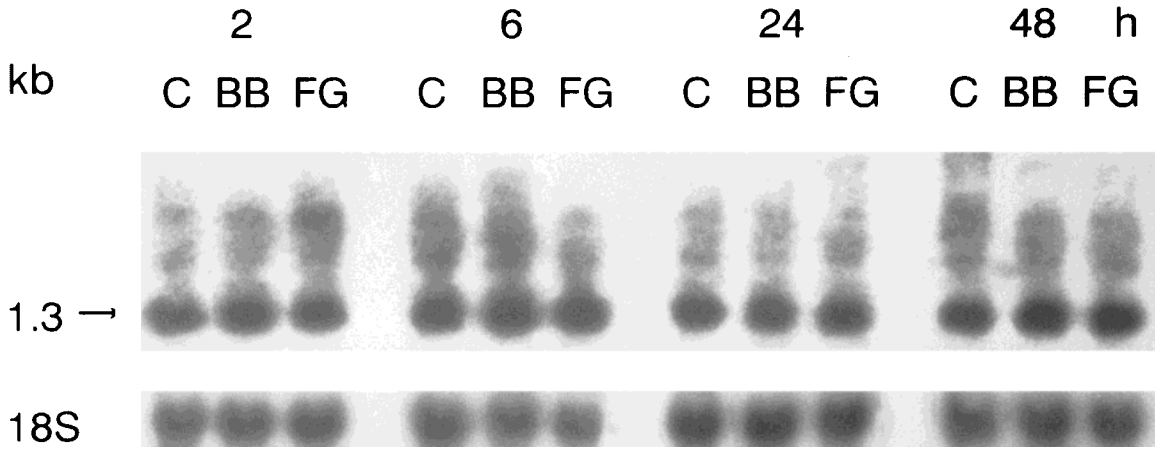

Fig. 7. Effect of platelet-derived growth factor BB (BB) at 3.3 nM and basic fibroblast growth factor (FG) at 6 nM on IGFBP-6 mRNA expression in cultures of Ob cells treated for 2, 6, 24, or 48 h. Total RNA from control (C) or treated cultures was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled rat IGFBP-6 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-6 mRNA was visualized by autoradiography and is shown in the **upper panel,** while 18S ribosomal RNA is shown in the **lower panel.**

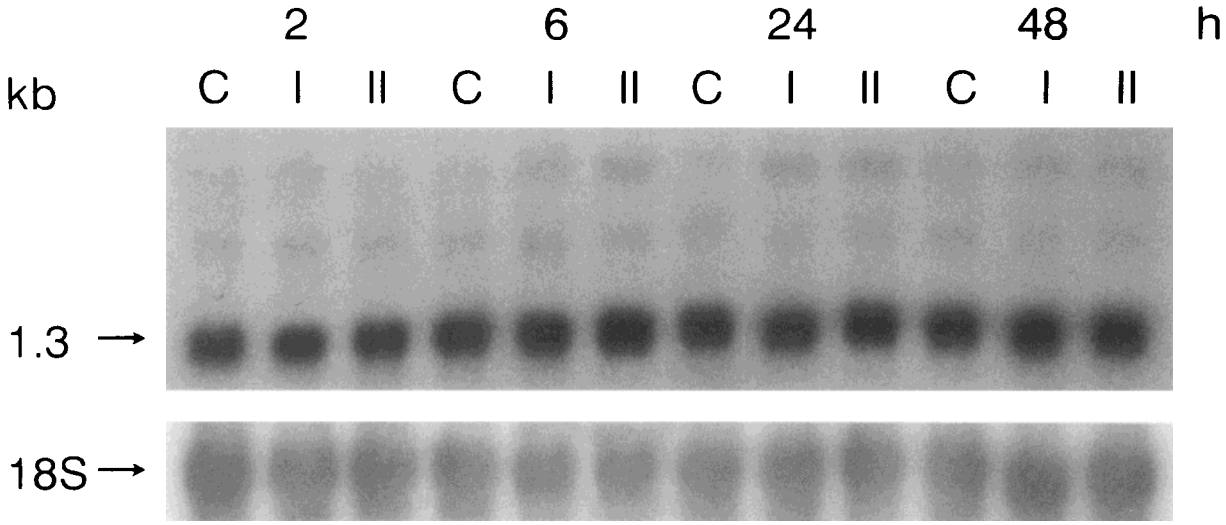

Fig. 8. Effect of insulin-like growth factors (IGF) I and IGF II on IGFBP-6 mRNA expression in cultures of Ob cells treated for 2, 6, 24, or 48 h. Total RNA from control (C) or IGF (I or II)-treated cultures was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled rat IGFBP-6 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. IGFBP-6 mRNA was visualized by autoradiography and is shown in the **upper panel,** while 18S ribosomal RNA is shown in the **lower panel.**

tion of IGFBP-6 by TGF β1 may facilitate IGF II actions and serve to maintain a physiological balance in the IGF/IGFBP axis. Although possibly acting through different mechanisms, exposure of the skeletal tissue to TGF β1 causes a biphasic effect on bone cell replication, an increase in type I collagen synthesis, and a decrease in interstitial collagenase expression [Centrella et al., 1987; Rydziel et al., 1997]. The effects on collagen and collagenase synthesis are similar to those of IGF II, and changes in bone matrix synthesis and degradation probably are the result of a fine balance among growth factors and their binding proteins in the bone microenvironment. In contrast, basic FGF and PDGF BB have potent mitogenic properties for bone cells, but they do not stimulate or even inhibit collagen synthesis and induce collagenase expression in osteoblasts [Canalis et al., 1988; Hock and Canalis, 1994; Varghese et al., 1995, 1996].

At the present time, there is no information about proteolytic enzymes and factors modifying either the degradation or distribution of

IGFBP-6 in skeletal tissue. In our initial studies, we found limited amounts of IGFBP-6 in extracellular matrix extracts, suggesting that there is limited distribution of IGFBP-6 to this site. TGF β1 could modify not only the synthesis but also the degradation of IGFBP-6 in bone cells, although we did not detect IGFBP-6 fragments in Ob cell cultures.

In conclusion, the present studies demonstrate that TGF β1 inhibits IGFBP-6 transcripts and polypeptide levels in skeletal cells through mechanisms that involve decreased transcription. The decreased level of IGFBP-6 in the bone microenvironment may increase the amount of bioavailable IGF II and be a feedback mechanism necessary for the maintenance of adequate levels of IGF II in skeletal tissue.

ACKNOWLEDGMENTS

The authors thank Dr. S. Shimasaki for the rat IGFBP-6 cDNA clone and antirat IGFBP-6 antibodies, Genentech for the gift of TGF β1, Eli Lilly Research Laboratories for the gift of IGF II, Ms. Cathy Boucher, Ms. Deena Durant, and Ms. Susan O'Lone for technical assistance, and Mrs. Margaret Nagle for secretarial help.

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