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Vitamin D_3 Metabolites Regulate LTBP1 and Latent TGF-β1 Expression and Latent TGF-β1 Incorporation in the Extracellular Matrix of Chondrocytes

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Abstract Growth plate chondrocytes make TGF-β1 in latent form (LTGF-β1) and store it in the extracellular matrix via LTGF-β1 binding protein (LTBP1). 1,25-$(OH)_2D_3$ (1,25) regulates matrix protein production in growth zone (GC) chondrocyte cultures, whereas 24,25-$(OH)_2D_3$ (24,25) does so in resting zone (RC) cell cultures. The aim of this study was to determine if 24,25 and 1,25 regulate LTBP1 expression as well as the LTBP1-mediated storage of TGF-β1 in the extracellular matrix of RC and GC cells. Expression of LTBP1 and TGF-β1 in the growth plate and in cultured RC and GC cells was determined by in situ hybridization using sense and antisense oligonucleotide probes based on the published rat LTBP1 and TGF-β1 cDNA sequences. Fourth passage male rat costochondral RC and GC chondrocytes were treated for 24 h with 10^{-7}-10^{-9} M 24,25 and 10^{-8}-10^{-10} M 1,25, respectively. LTBP1 and TGF-β1 mRNA levels were measured by in situ hybridization; production of LTGF-β1, LTGF-β2, and LTBP1 protein in the conditioned media was verified by immunoassays of FPLC-purified fractions. In addition, ELISA assays were used to measure the effect of 1,25 and 24,25 on the level of TGF-β1 in the media and matrix of the cultures. Matrix-bound LTGF-β1 was released by digesting isolated matrices with 1 U/ml plasmin for 3 h at 37°C. LTBP1 and TGF-β1 mRNAs are co-expressed throughout the growth plate, except in the lower hypertrophic area. Cultured GC cells express more LTBP1 and TGF-β1 mRNAs than RC cells. FPLC purification of the conditioned media confirmed that RC cells produce LTGF-β1, LTGF-β2, and LTBP1. GC cells also produce LTGF-β2, but at lower concentrations. 1,25 dose-dependently increased the number of GC cells with high LTBP1 expression, as seen by in situ hybridization. 24,25 had a similar, but less pronounced, effect on RC cells. 1,25 also caused a dose-dependent increase in the amount of TGF-β1 protein found in the matrix, significant at 10^{-8} and 10^{-9} M, and a corresponding decrease in TGF-β1 in the media. 24,25 had no effect on the level of TGF-β1 in the matrix or media produced by RC cells. This indicates that 1,25 induces the production of LTBP1 by GC cells and suggests that the TGF-β1 content of the media is reduced through the formation of latent TGF-β1-LTBP1 complexes which mediates storage in the matrix. Although 24,25 induced the expression of LTBP1 by RCs, TGF-β1 incorporation into the matrix is not regulated by this vitamin D_3 metabolite. Thus, vitamin D_3 metabolites may play a role in regulating the availability of TGF-β1 by modulating LTBP1 production. J. Cell. Biochem. 72:151–165, 1999.

Key words: Vitamin D_3, LTBP1, TGF-β, chondrocytes; latent TGF-β, 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$

Transforming growth factor beta (TGF-β) appears to play an important role in endochondral bone formation by regulating chondrocyte differentiation and maturation [Rosen et al., 1986, 1988]. This growth factor stimulates extracellular matrix synthesis by cartilage cells [Reed et al., 1994] and other connective tissue cells [Okuda et al., 1990; Bonewald and Mundy,

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1990; Broekelmann et al., 1991; Border et al., 1992]. Studies examining the response of growth plate chondrocytes to TGF-β indicate that the growth factor promotes chondrocyte maturation at early stages of differentiation by modulating proliferation and increasing alkaline phosphatase activity [Schwartz et al., 1993, 1996] and proteoglycan synthesis [Burton-Wurster and Lust, 1990; Pelton and Moses, 1990; Chen et al., 1991]. However, TGF-β also appears to inhibit terminal differentiation and matrix mineralization. TGF-β-dependent inhibition of matrix calcification has also been reported for osteoblast-like cell cultures [Bonewald and Mundy, 1990; Bonewald et al., 1990].

TGF-β1 is a member of the TGF-β superfamily of proteins and exists in a number of macromolecular forms. Preliminary studies indicate that the production of TGF-β1 by chondrocytes is very similar to that of bone cells. Most of the latent TGF-β1 (LTGF-β1) produced by bone and bone-like cells consists of a 100 kDa latent complex of the mature TGF-β1 homodimer and a Latency Associated Peptide (LAP) homodimer. A significant fraction of this latent homodimer forms a higher molecular weight macromolecule by associating through disulfide linkage to a 190 kDa protein, the Latent TGF-β1 Binding Protein-1 (LTBP1) [Kanzaki et al., 1990; Bonewald et al., 1991]. This high molecular weight (290 kDa) complex targets LTGF-β1 to the extracellular matrix of bone cells for storage [Dallas et al., 1995]. In preliminary studies we have found that cartilage cells, like bone cells, also produce the 290 kDa latent TGF-β1-LTBP1 macromolecule [Pedrozo et al., 1997]. One of the aims of the present study is to confirm this finding and to determine the major forms of the growth factor produced by chondrocytes at different stages of endochondral maturation.

Despite the similarities between cartilage and bone cells, most other cells possess tissue-specific differences in the relative amount of LTGF-β1 that is bound to LTBP1. For instance, platelets [Miyazono et al., 1988] and fibroblasts [Taipale et al., 1994] produce predominantly the latent TGF-β1-LTBP1 complex. Platelets produce a 130 kDa truncated form of LTBP1 [Miyazono et al., 1988]. Despite these differences, in all latent complexes, dissociation of mature TGF-β1 homodimer from the LAP is necessary for biological activity [Gentry et al., 1987; Lyons et al., 1988; Miyazono et al., 1988]. The LTBP1 molecule does not confer latency to TGF-β1, nor is it necessary for TGF-β1 processing or secretion [Gentry et al., 1988; Bonewald et al., 1991]. However, LTBP1 appears to be involved in bone matrix structure and formation, and it is also necessary for directing the 290 kDa latent complex to the extracellular matrix of bone cells for storage [Dallas et al., 1995]. The latter role provides a potential mechanism for the regulation of TGF-β1 activation.

We have previously shown that the metabolites of vitamin D_3, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, are important regulators of growth plate chondrocyte differentiation [Boyan et al., 1997]. Cellular response is determined by the maturational stage of the cell. 1,25-$(OH)_2D_3$ primarily affects growth zone (GC) cartilage cells [Boyan et al., 1988; Schwartz and Boyan, 1988], whereas 24,25-$(OH)_2D_3$ specifically targets resting zone (RC) cartilage cells. In the growth plate, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have complex feedback interactions [Boyan et al., 1997]. Adding another level of complexity, the interrelationship between the vitamin D_3 metabolites and TGF-β is also dependent on the maturational state of the cells. In RC cells, TGF-β1 downregulates 1,25-$(OH)_2D_3$ production and upregulates 24,25-$(OH)_2D_3$ production, but has no effect on either hydroxylase activity in GC cells [Schwartz et al., 1992; Pedrozo et al., 1996]. 1,25-$(OH)_2D_3$ decreases the content of TGF-β1 in the conditioned media of both RC and GC cells, but 24,25-$(OH)_2D_3$ has no effect on TGF-β1 release in either cell type [Boyan et al., 1994]. 24,25-$(OH)_2D_3$ and TGF-β1 exert a synergistic effect on the differentiation of RC cells, whereas in GC cells, the effects of TGF-β1 predominate over those of 1,25-$(OH)_2D_3$ [Schwartz et al., 1996, 1998].

These effects in cell culture reflect the interrelationships between RC and GC cells in the growth plate. RC cells synthesize extracellular matrix containing matrix vesicles with low levels of metalloproteinase activity [Dean et al., 1992]. As the cells mature and hypertrophy, the matrix is modified by the action of these enzymes. Matrix vesicles produced by GC cells exhibit higher levels of matrix-processing enzymes. Not surprisingly, matrix vesicles produced by GC cultures activate LTGF-β1 when incubated with 1,25-$(OH)_2D_3$, whereas matrix vesicles from RC cultures do not, whether 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ is used.

These observations led us to hypothesize that production of LTGF-β1 and its storage in the matrix by LTBP1 might be regulated in a cell maturation-dependent manner. The observation that LTBP1 forms a fibrillar matrix network in bone [Dallas et al., 1995] suggested that it might do so in cartilage as well. Extracellular matrix synthesis by growth plate chondrocytes is also sensitive to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in a cell maturation-dependent manner [Schwartz et al., 1989]. 1,25-$(OH)_2D_3$ stimulates collagen synthesis in GC cells, whereas 24,25-$(OH)_2D_3$ stimulates collagen synthesis in RC cells.

To test this hypothesis, we pursued three specific aims. First, we examined the mRNA expression levels of TGF-β1 and LTBP1 in cultures of RC and GC and in freshly dissected growth plate cartilage by in situ hybridization. Second, we sought to determine the production and secretion of different TGF-β isoforms as well as LTBP1 by RC and GC by examining the media conditioned by these cells. And, third, we determined if the latent TGF-β1-LTBP1 complex was targeted to the extracellular matrix for storage and the effect of vitamin D_3 metabolites on the production, secretion, and matrix-storage of this complex.

MATERIALS AND METHODS

Chondrocyte Culture

The chondrocyte culture system used in this study has been described in detail previously [Boyan et al., 1988, 1989, 1992]. Chondrocytes were isolated from the resting zone (RC) and growth zone (GC) cartilages of 125-g Sprague-Dawley rats and cultured at 37°C in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 50 μg/ml vitamin C in an atmosphere of 5% CO_2 and 100% humidity. Fourth passage cells were used for all experiments. Previous studies have shown that these cells retain their chondrocytic phenotype and differential responsiveness to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, as well as TGF-β, through four passages in culture [Boyan et al., 1988, 1989, 1992; Schwartz et al., 1993]. Previous studies demonstrated that chondrocyte response to the vitamin D metabolites is not gender-dependent [Ornoy et al., 1994]; therefore, male rats were used.

LTBP1 and TGF-β1 Gene Expression by In Situ Hybridization

In vitro. LTBP1 and TGF-β1 mRNA levels were determined by in situ hybridization. Confluent, third passage RC and GC chondrocytes were subcultured onto cover slips within 24-well plates. After 24 hours, the coverslips were transferred to new 24-well plates and the cells grown to subconfluency. At this point, in situ hybridization was performed according to a modification of the method described by Wilkinson [1992]. Reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. The coverslip was removed and the cells rinsed twice in 0.1 M phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 10 min at 4°C, and stored in 70% ethanol at −20°C, if necessary. The coverslips were rehydrated for 10 minutes in 0.1 M PBS, pre-treated with proteinase K (1 μg/ml diluted in 0.1 M Tris buffer, pH 3.0, 50 mM EDTA, pH 8.0), at 37°C for 10 min, postfixed in 4% paraformaldehyde, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, and rinsed in 2× SSC for 10 min. Hybridization was performed in a solution containing 50% formamide, 4× SSC, 10% dextran sulfate, 10 mM dithiothreitol (DTT), 500 μg/ml tRNA, 1× Denhardt's, and 100 μg/ml ssDNA. Thirty μl hybridization buffer containing the specific LTBP1 or TGF-β1 oligonucleotide probe with a specific activity of 1×10^6 cpm/μg was added to each coverslip in a humidified 24 well-plate and incubated overnight at 37°C. The coverslips were then washed with SSC containing 0.1 M β-mercaptoethanol as follows: 2 × SSC for 10 min at room temperature; 0.2 × SSC twice for 30 min at 52°C; 1 × SSC for 10 min at room temperature. Finally, the coverslips were air dried and then mounted face up on slides. All the solutions used were pretreated with 0.1% diethyl pyrocarbonate (DEPC) to destroy RNase activity.

To visualize the reaction, the coverslips were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with water, dried overnight at room temperature, and exposed at 4°C in desiccated slide boxes for 4 weeks. The exposed coverslips were developed in D-19 developer at 24°C for 4 min, fixed in Unifix for 5 min, and finally washed with water for 10 min. The samples were counterstained with 0.5% toluidine blue and mounted using Entellan

(Merck, Germany). All observations and photographs were made on an Aristoplan photomicroscope (Leitz, Wetzlar, Germany) using Kodak Ektar 100 film.

Sense and antisense oligonucleotide primers for LTBP1, corresponding to nucleotides 1591–1620 of the published rat LTBP1 cDNA sequence [Tsuji et al., 1990], and TGF-β1, corresponding to nucleotides 508–540 of the rat TGF-β1 sequence in GenBank (National Center for Biotechnology Information, NIH, Bethesda, MD), were synthesized in the University Protein Core facility using the 394 Synthesis Setup Listing (Version 2.01; Israel), checked for palindromes by GenBank data, labeled with [^{35}S]-DTP (specific activity of 4–5 $\times$ 10^8 cpm/μg) using the DNA 3'-End Labeling System (Promega, Madison, WI), and purified using Bio-Spin 6 chromatography columns (BIO-RAD, Hercules, CA). In both cases, sense probes were used as negative controls.

In vivo. The distribution of LTBP1 and TGF-β1 expression throughout the growth plate was determined by in situ hybridization. Tibias from Hebrew University Sabra strain rats were dissected, fixed in 4% paraformaldehyde in 0.1 M PBS, decalcified by incubation in 15% EDTA in 0.1 M PBS (pH 7.4) for 3 weeks, and embedded in paraffin. Longitudinal sections of 5 μm thickness were cut on a microtome and mounted on 3-aminopropyltriethoxysilane treated slides. Slides were stored at room temperature until ready to use, at which point the sections were de-waxed, fixed in 4% paraformaldehyde, and subjected to in situ hybridization for LTBP1 and TGF-β1 mRNA detection as explained above.

Latent TGF-β1 and LTBP1 Production and Characterization

Separation of latent TGF-β1, -β2, and LTBP1. To determine which latent forms of TGF-β and LTBP1 were produced and secreted by the chondrocytes, these proteins were purified by Fast Pressure Liquid Chromatography (FPLC) as previously described [Bonewald et al., 1991; Dallas et al., 1994; Marra et al., 1996]. Serum-free media conditioned by RC and GC cells were collected, concentrated, and fractionated by ion exchange chromatography. The LTBP1, LTGF-β1, and LTGF-β2 content of each fraction was determined by specific ELISAs. For these experiments, no attempt was made to determine the content of active TGF-β versus latent TGF-β in the fractions because the process of purification results in loss of active TGF-β; therefore, only latent TGF-β remained, all of which was acid-activated to permit its detection in the immunoassay. One liter of conditioned media from subconfluent fourth passage RC and GC chondrocytes grown in serum-free DMEM were collected and concentrated 100-fold using a Millipore concentrator with a 10,000 dalton cut-off membrane (Millipore, Bedford, MA). The concentrated media were dialyzed overnight against 20 mM Tris-HCl, pH 7.0 and serial dilutions of a small aliquot of the concentrate, 1/20 to 1/3,000, tested to determine total TGF-β content. Based on these dilutions, we estimated that 2 ml of concentrated media were sufficient to obtain detectable levels of protein after chromatographic fractionation. Two mls of concentrated media were applied to a Mono Q anion exchange column (Pharmacia, Piscataway, NJ) and fractionated using a linear 0 to 0.6 M NaCl gradient by mixing 20 mM Tris-HCl buffer, pH 7.0 (Buffer A) and 20 mM Tris-HCl buffer, pH 7.0, containing 1 M NaCl (Buffer B). The samples were eluted at 1 ml/min. Fractions were collected, concentrated to 400 μl with a Speed-Vac, and proteins precipitated by addition of 1 ml of ice cold 100% ethanol to reach a final concentration of 75% ethanol. Fractions were frozen overnight, centrifuged at 10,000*g* for 20 sec and the pellet resuspended in DMEM.

Immunoquantitation by ELISA. Specific ELISAs were used to measure TGF-β1, TGF-β2, and LTBP1 content of FPLC fractions as well as conditioned media and plasmin-released fractions as described below. To measure TGF-β1 or -β2, samples were acid-activated by addition of 10% volume of 1 M HCl, neutralized after 15 min by addition of equimolar amounts of NaOH, and immediately subjected to TGF-β1 (cat. # G1230, Promega, Madison, WI) or TGF-β2 ELISAs (R&D, Minneapolis, MN) according to the manufacturer's instruction.

LTBP1 ELISA was done according to Dallas et al. [1995] using Ab39, a rabbit polyclonal anti-rat LTBP1. All reagents were purchased from Sigma Chemical Company. Ninety-six-well plates were coated overnight at 4°C with 100 μl of standard or test sample diluted in PBS if necessary. The standard was run at 1 mg/ml followed by 1:1 serial dilutions thereof. Wells containing the samples and standard were washed 2$\times$ with 0.05% Tween 20 in PBS followed by blocking for 2 h at 37°C with 200 μl of

5% bovine serum albumin (BSA) in PBS and a wash. The wells were then incubated for 1 h at 37°C in 100 µl of a 1:1000 dilution of Ab39 in 0.5% BSA in PBS and washed six times to remove unbound Ab39. This was followed by incubation in the presence of 100 µl of the detection antibody (peroxidase-conjugated goat anti-rabbit IgG; 1:4,000 dilution) at 37°C for 1 h and repeated washing. The reaction was carried out by addition of 100 µl of o-phenylenediamine dihydrochloride (OPD) reagent for 10–30 min at 37°C in the presence of 1% hydrogen peroxide. Absorbance was determined at 450 nm.

Effect of Vitamin D_3 Metabolites on LTBP1 Expression

mRNA levels. The effect of 1,25-$(OH)_2D_3$ on GC and 24,25-$(OH)_2D_3$ on RC expression of LTBP1 mRNA was determined by in situ hybridization. Confluent, third passage RC and GC chondrocytes were subcultured onto coverslips, as previously described. When the cells reached subconfluence, they were treated with 10^{-9} or 10^{-8} M 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, respectively, for 24 h. In situ hybridization was performed as described above, followed by image analysis using the Gali system (Bet Haemek, Israel). The data were generated by counting the number of grains per cell in a sample of 50 randomly chosen cells per culture for a total of 300 cells per group, n = 6 cultures. Each cell was outlined and the number of grains determined within the area. This same outline was moved to a cell-free area adjacent to the cell in question and the number of grains counted to determine nonspecific binding. All images were evaluated by individuals "blinded" to the identity of the samples.

Release of latent TGF-β1-LTBP1 from the matrix. Fourth passage RC and GC cells were cultured to confluence in 24-well plates and treated for 24 h with 10^{-9} and 10^{-8} M 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, respectively. At harvest, the media were collected and a 50 µl aliquot was assayed for total TGF-β1 by ELISA. The cell layer was washed three times× with PBS, and the cells lysed by three successive 10-min washes with RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1% NP40, and 0.5% deoxycholate. The remaining matrix was washed three times with PBS and digested with 1.0 U/ml of plasmin in DMEM for 3 h at 37°C to release latent TGF-β1-LTBP1 complexes from the matrix as previously published [Dallas et al., 1994]. The reaction was stopped by addition of aprotinin to a final concentration of 5 mg/ml, and a 50 µl aliquot was assayed for total TGF-β1 by ELISA. Aprotinin, RIPA buffer, and plasmin were purchased from Sigma Chemical Company.

Statistical Management of the Data

Data are the mean ± SEM of six replicate cultures or samples (one sample/culture). Differences between treatment and control were assessed by Bonferroni's correction of Student's t-test, with significant differences among groups determined by values with $P < 0.05$.

RESULTS

LTBP1 and TGF-β1 Gene Expression

The expression of LTBP1 and TGF-β1 mRNA in the growth plate was demonstrated by the use of in situ hybridization (Fig. 1). A positive reaction when probed with the LTBP1 antisense oligonucleotide is observed in the prehypertrophic and upper hypertrophic, but not the lower hypertrophic zones of the growth plate (Fig. 1C). When probed with the antisense oligonucleotide for TGF-β1 (Fig. 1D), the growth plate showed a similarly positive reaction which colocalized with LTBP1 expression. Preparations probed with the sense oligonucleotide probe showed only background reactivity (Fig. 1B).

Both LTBP1 and TGF-β1 mRNA were also expressed in cultures of RC and GC cells. An example of GC cultures probed with TGF-β1 antisense or sense probes is shown in Figure 2. GC cells probed with LTBP1 and RC cells probed with TGF-β1 or LTBP1 had a qualitatively similar appearance (data not shown). Cultures of GC cells had higher TGF-β1 expression than cultures of RC cells (Fig. 3), as indicated by a higher proportion of RC cells with medium or no grains, and a higher proportion of GC cells with greater than 20 grains per cell. In a similar fashion, LTBP1 expression was evaluated and found to be slightly higher in GC than RC cells (Fig. 4).

Latent TGF-β1 and LTBP1 Protein Production

FPLC separation of media conditioned by cultured RC cells indicates that these cells produce both latent TGF-β1 and latent TGF-β2 with 4× more β1 than β2 isoform (Fig. 5A). Two

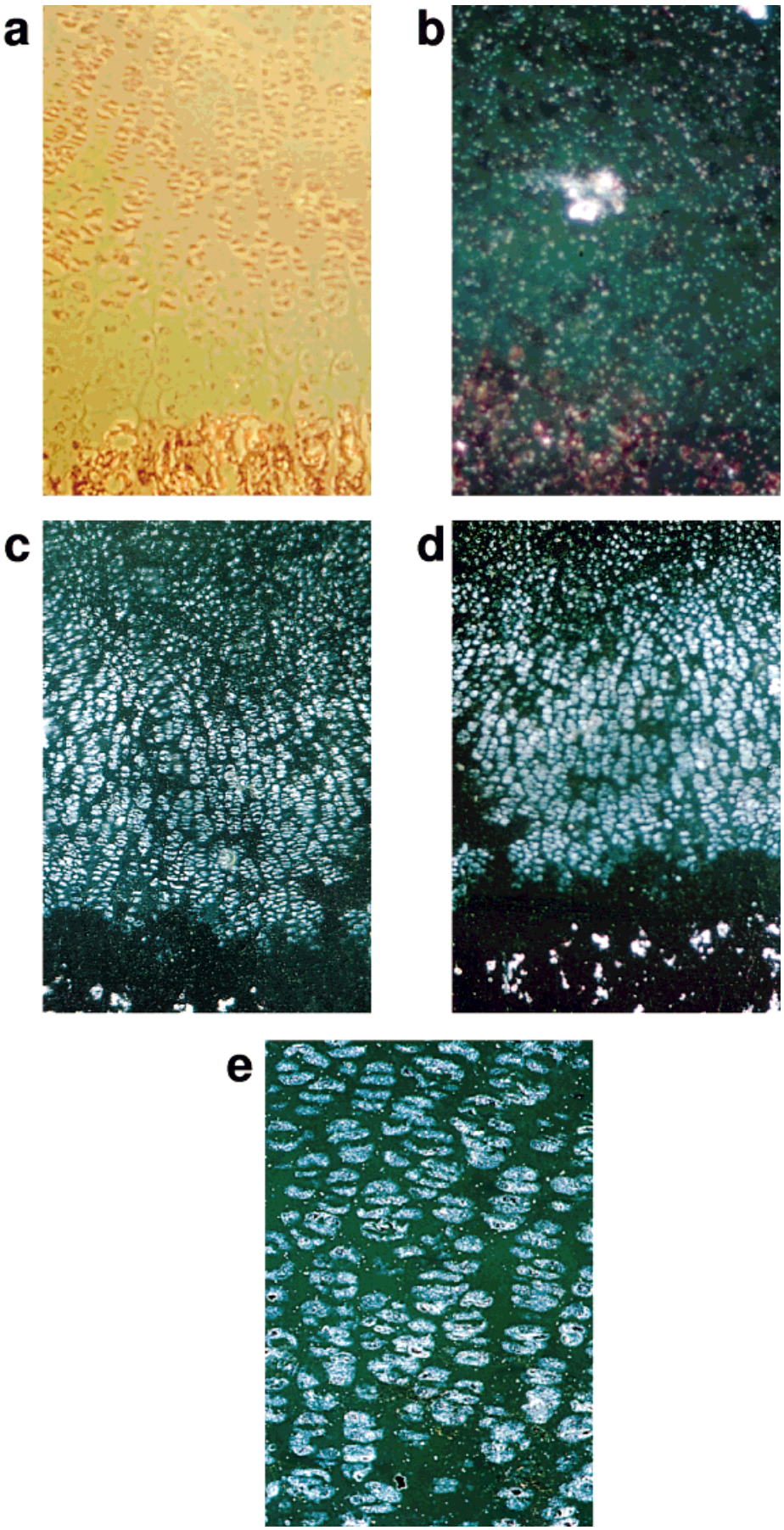

Figure 1.

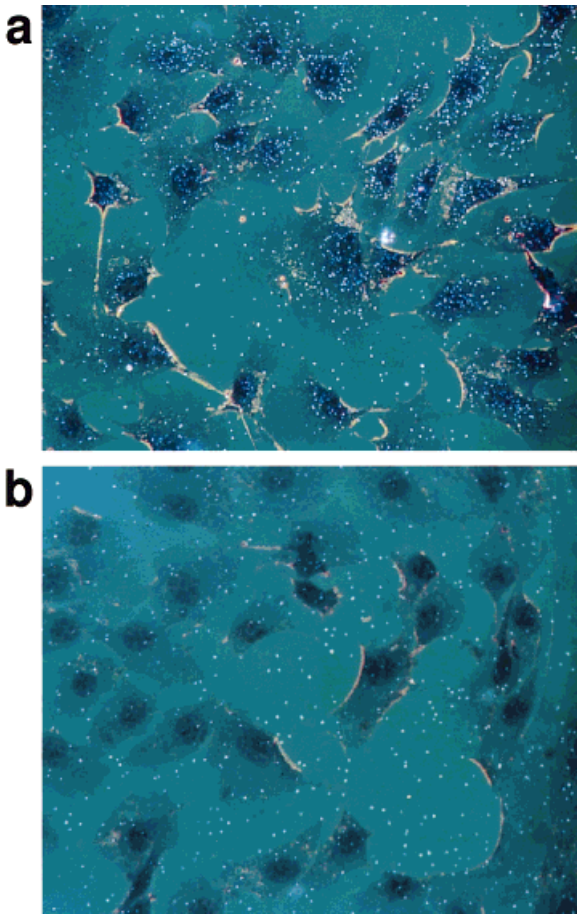

Fig. 2. In situ hybridization showing TGF-β1 mRNA expression by cultured growth zone (GC) chondrocytes. A 33bp antisense oligonucleotide probe (corresponding to nucleotides 508 -540 of rat TGFβ1) was used to probe subconfluent cultures of GC (**A**). A 33bp sense oligonucleotide probe to the same sequence was used as a negative control (**B**). Original maginification is 100×.

major peaks containing TGF-β1 immunoreactivity were present, one eluting at approximately 0.2 M NaCl and the second one at approximately 0.35 M NaCl. The profile for LTBP1 shows a single peak occurring at 0.35 M NaCl

Fig. 1. In situ hybridization showing LTBP1 and TGF-β1 mRNA expression in the rat costochondral growth plate. **A:** A light photomicrograph of the growth plate with the resting zone in the upper portion and the mineralizing front in the lower portion of the panel. Oligonucleotide primers for LTBP1 (corresponding to nucleotides 1591–1620) and TGFβ1 (corresponding to nucleotides 508–540) were synthesized and used to detect the presence and distribution of mRNA for these molecules in growth plate. **C,D:** In situ hybridization for antisense oligonucleotide probes for LTBP1 and TGFβ1, respectively. Sense oligonucleotides were used as negative controls for LTBP1 (**B**) and TGFβ1 (data not shown). **E:** A higher magnification of C. Original magnification of A–D is 100× and E is 400×.

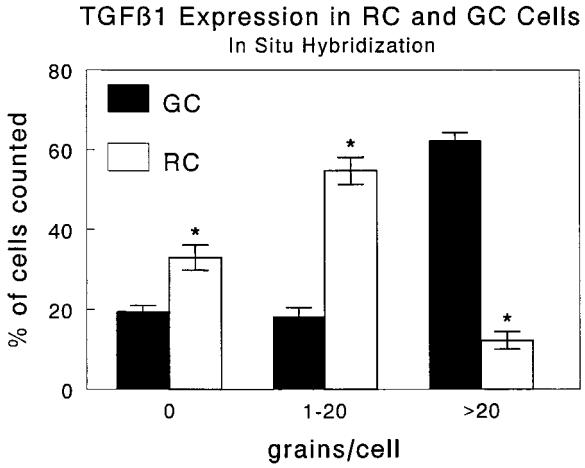

Fig. 3. Assessment of TGF-β1 expression in resting zone (RC) and growth zone (GC) chondrocytes by in situ hybridization. Fourth passage, subconfluent chondrocytes were hybridized with a 33bp oligonucleotide probe corresponding to nucleotides 508 to 540 of rat TGF- β 1 cDNA. The primer was labeled with [^{35}S]-Dtp using the DNA 3'-End Labeling System (Promega, Madison, WI). The number of grains per cell was counted as described in Materials and Methods and the results expressed as the percent of cells with no grains, 1–20 grains, or greater than 20 grains per cell. Values represent the mean ± SEM for n = 6 individual cultures in a representative experiment. *$P < 0.05$, GC versus RC using the Student's t-test.

and overlapping in shape and elution time with the second TGF-β1 immunoreactive peak (Fig. 5B). The production of latent TGF-β2 is less in cultured GC cells compared to RC cells; however, the profile also shows two major peaks of the latent TGF-β1 isoform, with very similar elution times to that observed in RC cells (Fig. 6A). The LTBP1 profile also showed a single peak overlapping in shape and elution time with the 0.35 M NaCl TGF-β1 immunoreactive peak (Fig. 6B).

Effect of Vitamin D_3 Metabolites on LTBP1 and TGF-β1 Expression

Treatment of RC cells with 24,25-$(OH)_2D_3$ had no effect of TGF-β1 mRNA expression (Fig. 7). Regardless of the concentration of the metabolite used, these cultures had a similar pattern of TGF-β1 mRNA expression to that observed in untreated cultures (Fig. 3). In contrast, 1,25-$(OH)_2D_3$ caused an increase in TGF-β1 mRNA levels in GC cells at the higher dose (Fig. 8). This was indicated by a shift in the number of cells with 1–20 grains per cell to greater than 20 grains per cell when treated with 10^{-8} M 1,25-$(OH)_2D_3$. LTBP1 expression in

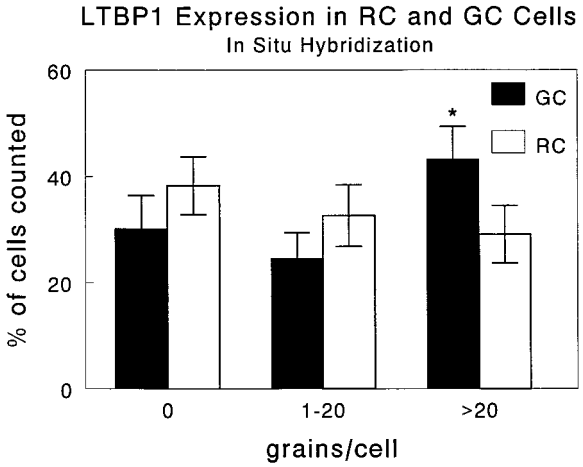

Fig. 4. Assessment of LTBP1 expression in resting zone (RC) and growth zone (GC) chondrocytes by in situ hybridization. Fourth passage, subconfluent chondrocytes were hybridized with a 30bp oligonucleotide probe corresponding to nucleotides 1591 to 1620 of rat LTBP1 cDNA. The primer was labeled with [^{35}S]-Dtp using the DNA 3'-End Labeling System (Promega, Madison, WI). The number of grains per cell was counted as described in Materials and Methods and the results expressed as the percent of cells with no grains, 1–20 grains, or greater than 20 grains per cell. Values represent the mean ± SEM for n = 6 individual cultures in a representative experiment. *$P < 0.05$, GC versus RC using the Student's t-test.

RC cells was significantly increased by 24,25-$(OH)_2D_3$, but the effect was not pronounced (Fig. 9). In contrast, GC cultures exhibited a more definitive response to 1,25-$(OH)_2D_3$ (Fig. 10). There was a dose-dependent shift in the number of cells with 1–20 grains per cell to greater than 20 grains per cell in the cultures treated with 10^{-8} M 1,25-$(OH)_2D_3$.

Effect of Vitamin D_3 Metabolites on TGF-β1 Production or Storage in the Matrix

24,25-$(OH)_2D_3$ had no effect on either total TGF-β1 in the media or on the storage of latent TGF-β1 in the extracellular matrix of RC cells (Fig. 11). In contrast, GC cells responded to treatment with 10^{-9} and 10^{-8} M 1,25-$(OH)_2D_3$ by reducing the amount of total TGF-β1 in the media and increasing its extracellular matrix content in a dose-dependent manner (Fig. 12).

DISCUSSION

In this study, we show that RC and GC chondrocytes both produce latent TGF-β1 and latent TGF-β2, although cell maturation-dependent differences exist in the total amounts of each protein produced by the cells. In comparison with RC cells, GC cells produce more latent TGF-β1 and less latent TGF-β2. Although we did not measure TGF-β production in a quantitative manner, based on semiquantitative ELISAs, GCs produced more than twice as much TGF-β1 as did RC cells. Previously, we used antibodies to TGF-β1 and TGF-β2 to deplete these isoforms from RC and GC conditioned media and found that GC produced both TGF-β1 and TGF-β2, whereas RCs produced only TGF-β1 [Boyan et al., 1994]. Moreover, the amount of TGF-β1 and TGF-β2 produced by GC and RC cells was roughly equivalent. In this earlier study, we used a bioassay based on stimulation of alkaline phosphatase to detect the protein, which may account for some of the discrepancy between the two sets of data, since alkaline phosphatase is sensitive to other components in serum, including 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. In addition, total TGF-β was measured in the bioassay, whereas in the present study, only latent growth factor was measured. Despite these differences, the two data sets agree very well in the amount of TGF-β1 and TGF-β2 produced. The bioassay indicated that GC cells produce approximately 10 ng/ml total TGF-β1 and 2 ng/ml total TGF-β2, and RC cells produce approximately 7.5 ng/ml TGF-β1 and 2.2 ng/ml TGF-β2. The immunoassays in the present study indicated that GC cells produce approximately 9.2 ng/ml LTGF-β1 and 0.3 ng/ml LTGF-β2, while RC cells produce 7.7 ng/ml LTGF-β1 and 0.5 ng/ml LTGF-β2.

The physiological significance of the differential production of TGF-β1 and TGF-β2 by GC and RC cells is not clear. TGF-β1 has been shown in a number of studies to regulate cell proliferation and extracellular matrix production; early stages of differentiation are stimulated, but terminal differentiation is blocked [Rosen et al., 1988; Bonewald et al., 1992; Caplan and Boyan, 1994]. Far less is known concerning TGF-β2. This isoform appears to modulate proliferation [Zerath et al., 1997] and may have a role in growth plate differentiation as well.

The results of this study confirm that costochondral growth plate chondrocytes produce a high molecular weight form of the TGF-β1 complex [Pedrozo et al., 1997]. This high molecular weight form appears to result from the association of the latent TGF-β1 homodimer to the LTBP1 molecule. This is indicated by the overlap in profile and elution time of the second TGF-β1 peak with the major peak in the LTBP1

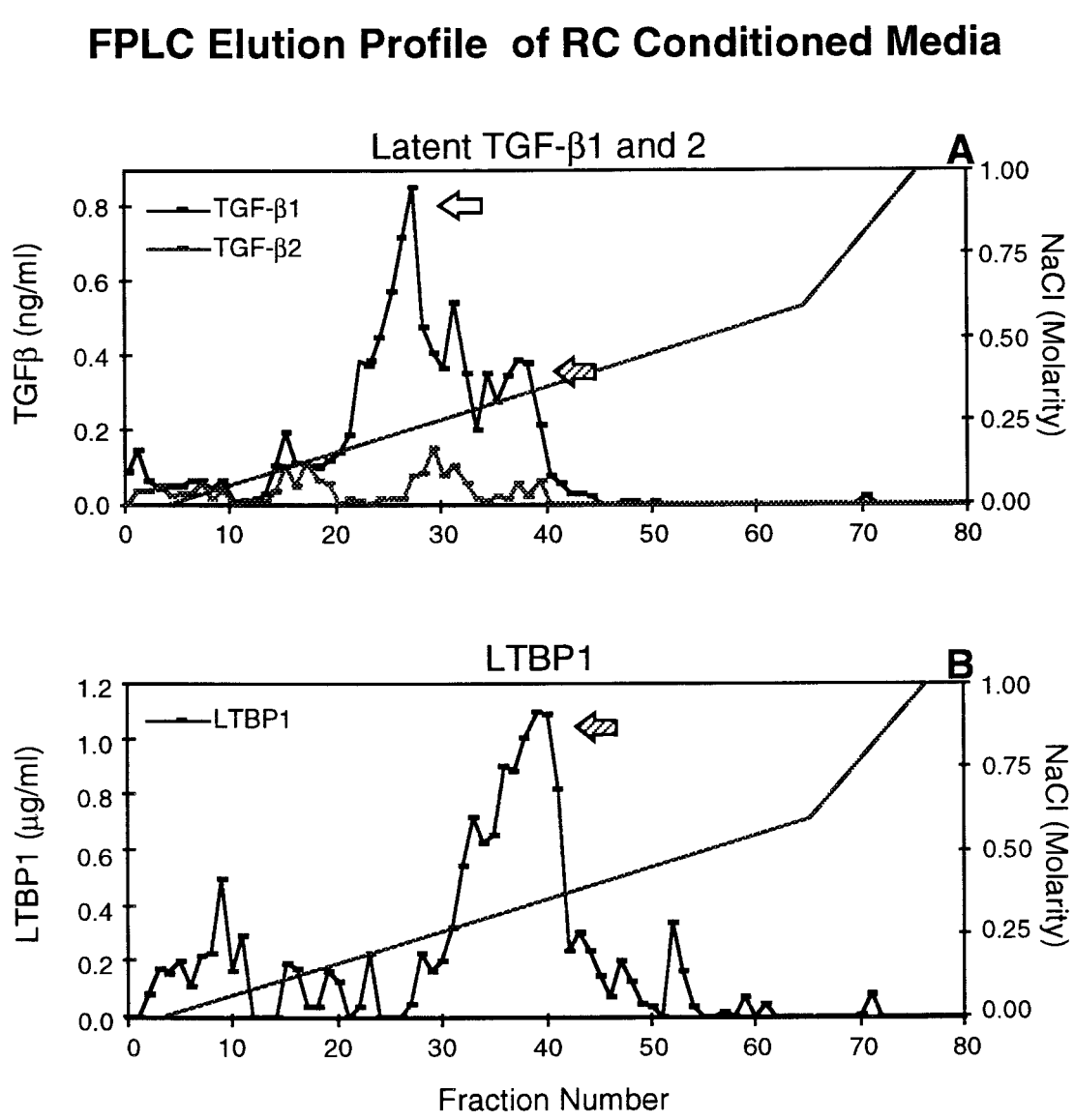

Fig. 5. FPLC elution profile of media conditioned by resting zone (RC) chondrocytes. One liter of conditioned media was concentrated to 10 ml, dialyzed against 20 mM Tris-HCl, pH 7.0, and fractionated on a Mono Q anionic column using a 0–1.0 M NaCl gradient. The collected fractions were concentrated, resuspended in DMEM, and aliquots assayed for their content of TGF-β 1, -β 2, and LTBP1 by ELISA. **A:** Shows the TGF-β1 and β2 content per fraction. **B:** Shows the LTBP1 content per fraction.

FPLC profile. It is likely that the early-eluting TGF-β1 peak corresponds to the 100 kDa TGF-β1 LAP homodimer, based on the similarities with bone TGF-β1 production [Bonewald et al., 1991; Dallas et al., 1994]. Bone cells produce two major complexes of TGF-β1 with similar elution times, the first one corresponding to the latent homodimer and the second one to the 290 kDa latent complex in association with the LTBP1 protein [Bonewald et al., 1991].

As seen in chondrocytes, bone cells also produce TGF-β1 and -β2 in a ratio of about 9 to 1. In contrast, fibroblasts and liver cells only produce TGF-β1 and TGF-β1 associated with the full length 190 kDa form of the LTBP1 molecule [Bonewald et al., 1991]. A further variation is found in platelets where TGF-β1 is the only isoform produced, but is also found associated with a truncated form of LTBP1 with a molecular weight of 130 kDa [Miyazono et al., 1988]. Other cell types produce higher proportions of TGF-β2. Kidney mesangial cells produce TGF-β1 and -β2 in a 1:1 ratio [Marra et al., 1996] and prostate cells produce up to 10 times the amount of TGF-β1 compared to TGF-β2 [Zhao et al., 1997]. These tissue-specific differ-

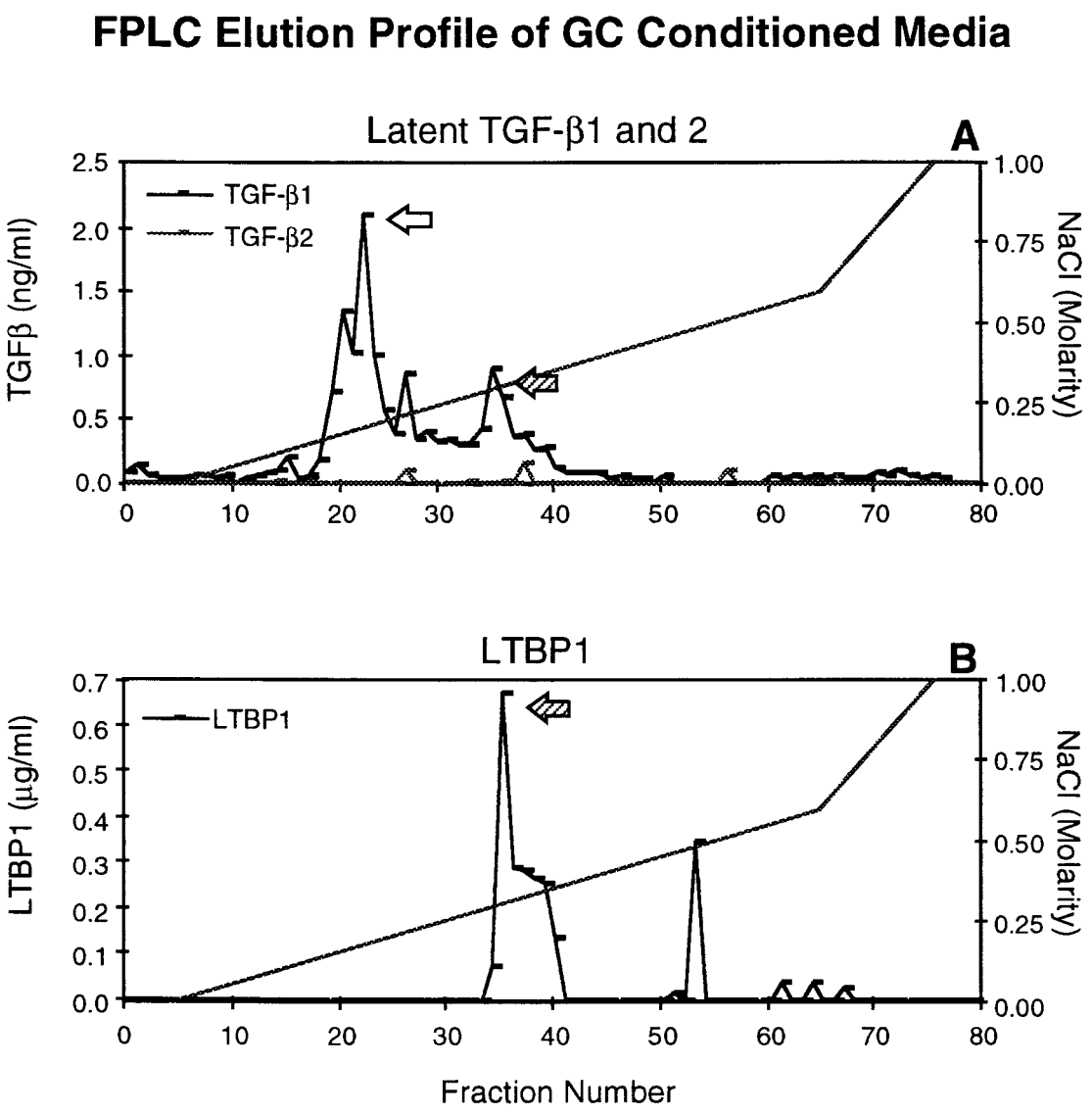

Fig. 6. FPLC elution profile of media conditioned by growth zone (GC) chondrocytes. One liter of conditioned media was concentrated to 10 ml, dialyzed against 20 mM Tris-HCl, pH 7.0, and fractionated on a Mono Q anionic column using a 0–1.0 M NaCl gradient. The collected fractions were concentrated, resuspended in DMEM, and aliquots assayed for their content of TGF-β1, -β2, and LTBP1 by ELISA. **A:** Shows the TGF-β1 and β2 content per fraction. **B:** Shows the LTBP1 content per fraction.

ences in the ratio of TGF-β1 to TGF-β2 and TGF-β to LTBP1 suggest specific roles for TGF-β2 and LTBP1.

The hypothesis that latent TGF-β1 is complexed with LTBP1 is supported by the co-expression of the mRNAs for these proteins in the RC and GC of the growth plate in vivo. The same cells that express mRNA for TGF-β1 also express mRNA for LTBP1 both in vivo and in culture. Moreover, the greatest expression based on fluorescence intensity in vivo and on grain counts in vitro was in GC cells (prehypertrophic and upper hypertrophic zones). This correlates with the greater production of LTGF-β1 and LTBP1 by GC cells indicated by FPLC purification of the conditioned media and extracellular matrix content of the LTGF-β1-LTBP1 complex. Similarly, Jingushi et al. [1990] noted that TGF-β1 was localized in the cells and extracellular matrix of the prehypertrophic and upper hypertrophic zones, but not the mineralizing front of rat epiphyseal growth plate.

This spatial distribution of TGF-β1 and its binding protein may be related to its function in

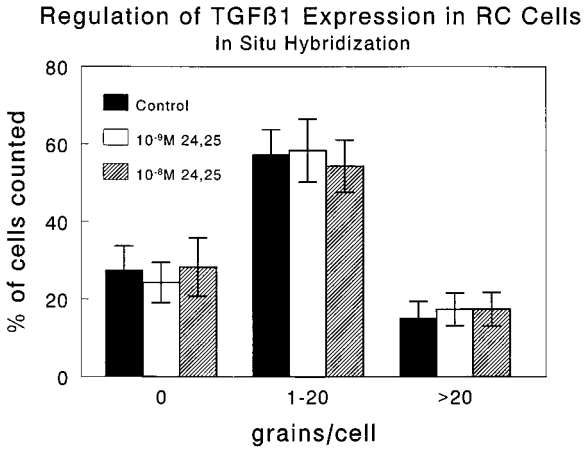

Fig. 7. Effect of 24,25-$(OH)_2D_3$ on TGF-β1 mRNA levels in resting zone (RC) cells. Fourth passage, subconfluent RC chondrocytes were treated with 10^{-9} and 10^{-8} M 24,25-$(OH)_2D_3$ for 24 h, and at harvest, the cultures were hybridized with a 33bp oligonucleotide probe corresponding to nucleotides 508–540 of rat TGF-β1 cDNA. The primer had been previously labeled with [^{35}S]-Dtp using the DNA 3'-End Labeling System (Promega, Madison, WI). The number of grains per cell was counted as decribed in Materials and Methods and the results expressed as the percent of cells with no grains, 1–20 grains, or greater than 20 grains per cell. Values represent the mean ± SEM for n = 6 individual cultures in a representative experiment. *$P < 0.05$, treatment versus control using the Student's t-test.

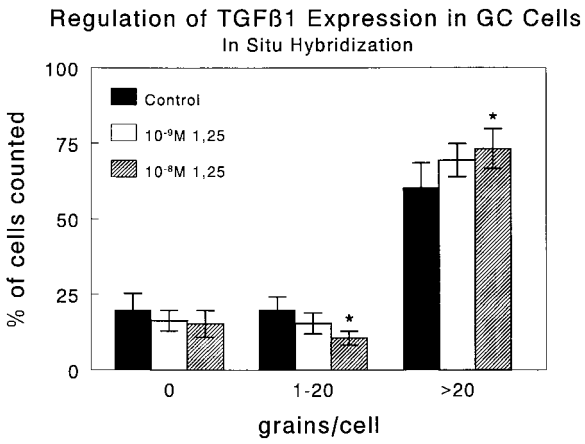

Fig. 8. Effect of 1,25-$(OH)_2D_3$ on TGF-β1 mRNA levels in growth zone (GC) cells. Fourth passage, subconfluent GC chondrocytes were treated with 10^{-9} and 10^{-8} M 1,25-$(OH)_2D_3$ for 24 h, and at harvest, the cultures were hybridized with a 33bp oligonucleotide probe corresponding to nucleotides 508–540 of rat TGF-β1 cDNA. The primer was labeled with [^{35}S]-Dtp using the DNA 3'-End Labeling System (Promega, Madison, WI). The number of grains per cell was counted as described in Materials and Methods and the results expressed as the percent of cells with no grains, 1–20 grains, or greater than 20 grains per cell. Values represent the mean ± SEM for n = 6 individual cultures in a representative experiment. *$P < 0.05$, treatment versus control using the Student's t-test.

the growth plate. TGF-β1 has been shown to inhibit phospholipase A_2 activity in both RC and GC cells; however, in the maturing growth plate, particularly in the lower hypertrophic cell zone, this enzyme activity is increased [Wuthier, 1973]. This increase is associated with the breakdown of matrix vesicles and calcium phosphate deposition in the matrix. This suggests that TGF-β1 has an anti-mineralization effect. A downregulation of TGF-β1 expression in the mineralizing, lower hypertrophic zone would permit mineralization and bone formation to occur.

Our results indicate that the cells produce relatively high levels of TGF-β1, approximately 9.2 ng/ml for GC cells and 7.6 ng/ml for RC cells, yet both GC and RC cells exhibit most of their biological responses to TGF-β1 concentrations from 0.1 to 0.22 ng/ml, well below the levels needed for osteoblasts to register a comparable effect [Bonewald et al., 1990]. The range of concentrations to which chondrocytes are responsive is extremely narrow. These observations suggest that the relationship of LTGF-β1 with its binding protein is critical in maintaining active TGF-β1 at appropriate concentrations for optimal cell response.

Even though GC cells express more TGF-β1 protein and LTBP1 message and protein than RC cells, the latter produce twice as much of the high molecular weight latent TGF-β1-LTBP1 complex as indicated by FPLC. This is in sharp contrast to cultured bone and bone-like cells [Dallas et al., 1994], which have a production profile that is similar to that of GC cells. In the data presented here and in our previous report [Pedrozo et al., 1997], there were no apparent differences between the amount of latent TGF-β1 stored in the extracellular matrix of RC and GC cells. This discrepancy between the amounts of latent TGF-β1-LTBP1 secreted into the media and the amount of latent TGF-β1 stored in the extracellular matrix indicate that the association of this growth factor to the LTBP1 molecule may not be the sole determinant for the extracellular storage of latent TGF-β1. It also indicates that the association of TGF-β1 to LTBP1 may serve a purpose distinct from the extracellular matrix storage of this growth factor. And most importantly, it indicates that the association of latent TGF-β1-LTBP1 to the extracellular matrix of chondrocytes is cell maturation-dependent.

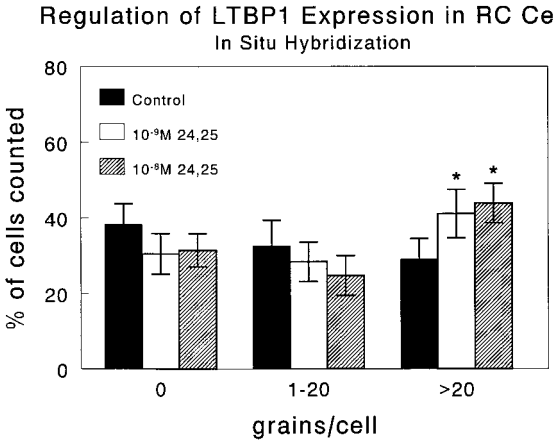

Fig. 9. Effect of 24,25-$(OH)_2D_3$ on LTBP1 mRNA levels in resting zone (RC) cells. Fourth passage, subconfluent RC chondrocytes were treated with 10^{-9} and 10^{-8} M 24,25-$(OH)_2D_3$ for 24 h, and at harvest, the cultures were hybridized with a 30bp oligonucleotide probe corresponding to nucleotides 1591–1620 of LTBP1. The primer was labeled with [^{35}S]-Dtp using the DNA 3'-End Labeling System (Promega, Madison, WI). The number of grains per cell was counted as described in Materials and Methods and the results expressed as the percent of cells with no grains, 1–20 grains, or greater than 20 grains per cell. Values represent the mean ± SEM for n = 6 individual cultures in a representative experiment. *$P < 0.05$, treatment versus control using the Student's t-test.

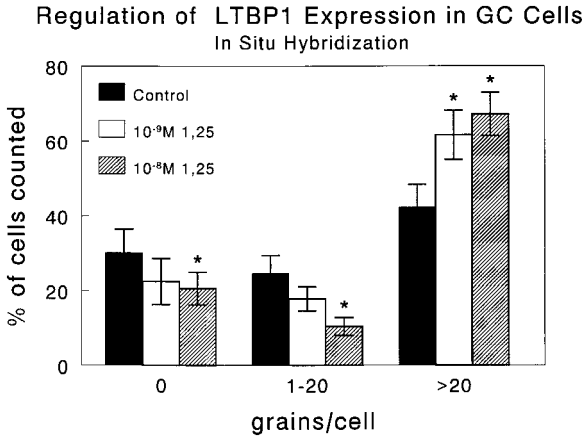

Fig. 10. Effect of 1,25-$(OH)_2D_3$ on LTBP1 mRNA levels in growth zone (GC) cells. Fourth passage, subconfluent GC chondrocytes were treated with 10^{-9} and 10^{-8} M 1,25-$(OH)_2D_3$ for 24 h, and at harvest, the cultures were hybridized with a 30bp oligonucleotide probe corresponding to nucleotides 1591–1620 of LTBP1. The primer was labeled with [^{35}S]-Dtp using the DNA 3'-End Labeling System (Promega, Madison, WI). The number of grains per cell was counted as described in Materials and Methods and the results expressed as the percent of cells with no grains, 1–20 grains, or greater than 20 grains per cell. Values represent the mean ± SEM for n = 6 individual cultures in a representative experiment. *$P < 0.05$, treatment versus control using the Student's t-test.

Regulation of TGF-β and LTBP1 expression in RC cells by 24,25-$(OH)_2D_3$ and in GC cells by 1,25-$(OH)_2D_3$ was cell-specific. As noted previously [Boyan et al., 1994], 24,25-$(OH)_2D_3$ had no effect on TGF-β1 mRNA levels. In contrast, 24,25-$(OH)_2D_3$ increased expression of LTBP1.

GC cells responded to treatment with 1,25-$(OH)_2D_3$ by increasing both LTBP1 and TGF-β1 message. The sensitivity of the in situ hybridization technique enabled us to observe the subtle effect of 1,25-$(OH)_2D_3$ on TGF-β1 mRNA levels previously undetected by the use of Northern blot analysis [Boyan et al., 1994]. The amount of latent TGF-β1 stored in the extracellular matrix of GC cultures also increased after treatment with 1,25-$(OH)_2D_3$. This increase in matrix-associated latent TGF-β1 correlated well with the 1,25-$(OH)_2D_3$-dependent decrease in TGF-β1 in the GC-conditioned media noted in previous studies [Boyan et al., 1994].

Taken together with the increase in LTBP1 message, these data suggest that in the more mature chondrocytes, 1,25-$(OH)_2D_3$ regulates total TGF-β1 secretion by regulating the extracellular storage of the growth factor from gene transcription to the protein targeting level. These data explain our previous finding in which 1,25-$(OH)_2D_3$ decreased total TGF-β1 in the media [Boyan et al., 1994]. These results are in contrast to a report by Farquharson et al. [1996] in which they found that in cultured chick growth plate, chondrocytes increased total TGF-β1 mRNA and media content at the same dose observed in our study. This may be due to species-specific differences and the heterogeneity of their cell preparation, since they do not distinguish between the more and less mature chondrocytes.

In contrast to the GC cultures, RC cultures treated with 24,25-$(OH)_2D_3$ increased LTBP1 expression without a change in TGF-β1 expression. Similar to our previous data [Boyan et al., 1994], there was no detectable effect of 24,25-$(OH)_2D_3$ on total TGF-β1 in the media nor in the matrix of RC cells also showing that, as in bone cells, TGF-β1 and LTBP1 can be regulated separately [Dallas et al., 1994].

The maturation-dependent differences in TGF-β1 and LTBP1 mRNA levels and protein production between RC and GC cells in response to their metabolite of choice suggests that 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ may regulate LTBP1 production for different purposes. This is supported by the dual role of LTBP1. In

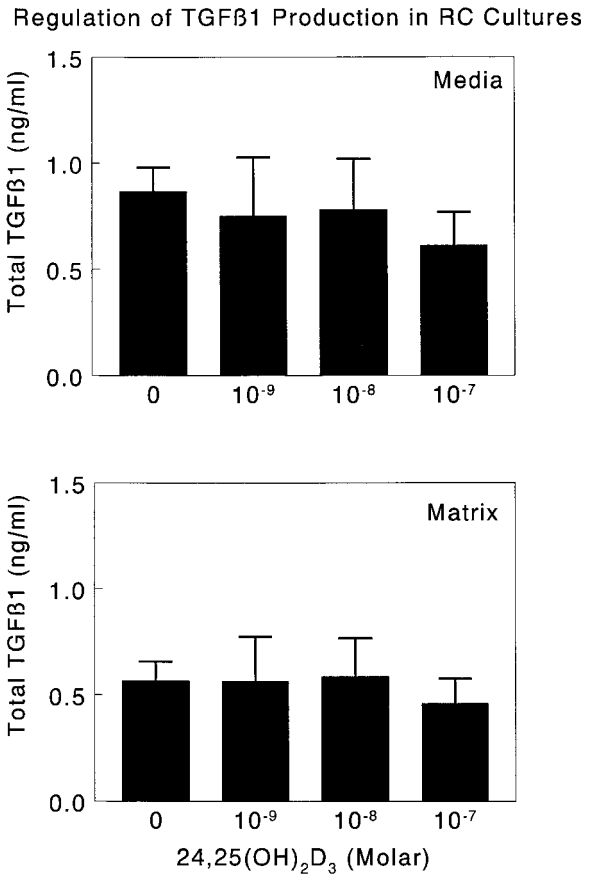

Fig. 11. Effect of 24,25-$(OH)_2D_3$ on the level of TGF-β1 in the media and extracellular matrix of resting zone (RC) cells. Subconfluent, fourth passage RC chondrocytes were treated with 10^{-9}-10^{-7} M 24,25-$(OH)_2D_3$ for 24 h. The media were collected and the extracellular matrices digested with 1 U/ml of plasmin for 3 h to release TGF-β1. Total TGF-β1 in the media (upper panel) and plasmin digests of the matrix (lower panel) were measured by ELISA. Values represent the mean ± SEM for n = 6 samples in a single experiment. *$P < 0.05$, treatment versus control using the Student's t-test.

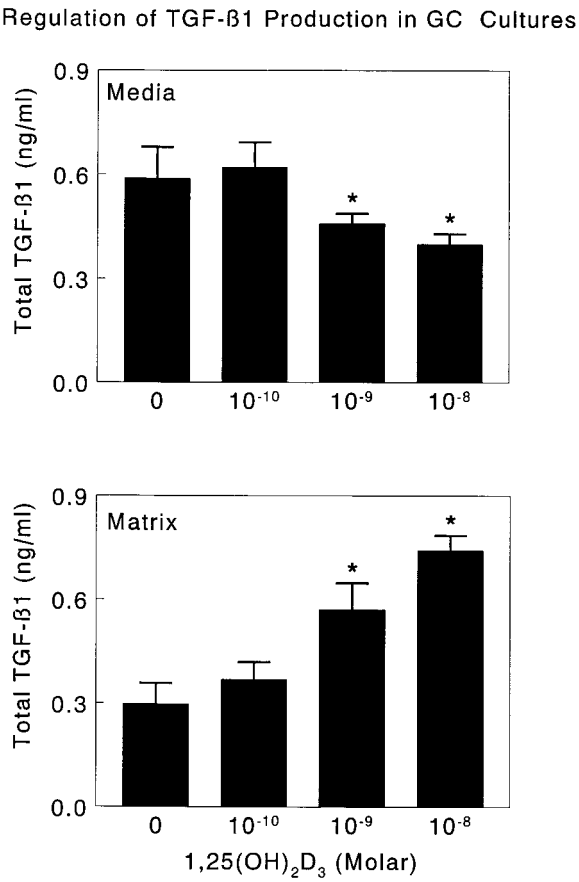

Fig. 12. Effect of 1,25-$(OH)_2D_3$ on the level of TGF-β1 in the media and extracellular matrix of growth zone (GC) cells. Subconfluent, fourth passage GC chondrocytes were treated with 10^{-10}-10^{-8} M 1,25-$(OH)_2D_3$ for 24 h. The media were collected and the extracellular matrices digested with 1 U/ml of plasmin for 3 h to release TGF-β1. Total TGF-β1 in the media (upper panel) and plasmin digests of the matrix (lower panel) were measured by ELISA. Values represent the mean ± SEM for n = 6 samples in a single experiment. *$P < 0.05$, treatment versus control using the Student's t-test.

addition to mediating the extracellular storage of latent TGF-β1, LTBP1 has been shown to act as a structural protein necessary for nodule formation in cultures of fetal rat calvarial cells [Dallas et al., 1995]. It is possible that 24,25-$(OH)_2D_3$ increases LTBP1 production to modulate extracellular matrix formation in RC cells and that 1,25-$(OH)_2D_3$ does the same in GC cells, but with a different goal, to augment the storage of latent TGF-β1 in the extracellular matrix.

In the past, we showed that matrix vesicles, extracellular organelles rich in neutral metalloproteinases, activate latent TGF-β upon treatment with 1,25-$(OH)_2D_3$ [Boyan et al., 1994]. The results presented here further demonstrate that the extracellular matrix is an important site for the regulation of TGF-β1. While stored in the matrix, latent TGF-β1 is made available to matrix vesicles for activation. The release and activity of the matrix vesicle enzymes is under genomic and nongenomic regulation by chondrocytes; therefore, the cells can regulate the temporal and spatial activation of latent TGF-β1 at remote sites from the cells by regulating the content and function of matrix vesicles. By the same token, the amount of latent TGF-β1 stored in the extracellular matrix and made available to matrix vesicles is also under genomic and nongenomic regulation. This adds another dimension to the complex modulation of latent TGF-β1 and the avail-

ability of this important growth factor when and where it is needed within the growth plate.

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