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Accumulation, Localization, and Compartmentation of Transforming Growth Factor β During Endochondral Bone Development

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Abstract. Endochondral bone formation was induced in postnatal rats by implantation of demineralized rat bone matrix. Corresponding control tissue was generated by implanting inactive extracted bone matrix, which did not induce bone formation. At various times, implants were removed and sequentially extracted with guanidine hydrochloride, and then EDTA and guanidine hydrochloride. Transforming growth factor β (TGFβ) in the extracts was quantitated by a radioreceptor assay. TGFβ was present in demineralized bone matrix before implantation, and the concentration had decreased by 1 d after implantation. Thereafter, TGFβ was undetectable by radioreceptor assay until day 9. From day 9–21 the TGFβ was extracted only after EDTA demineralization, indicating tight association with the mineralized matrix. During this time, the content of TGFβ per milligram soluble protein rose steadily and remained high through day 21. This increased concentration correlated with the onset of vascularization and calcification of cartilage. TGFβ was detected only between days 3–9 in the controls; i.e., non-bone-forming implants. Immunolocalization of TGFβ in bone-forming implants revealed staining of inflammatory cells at early times, followed later by staining of chondrocytes in calcifying cartilage and staining of osteoblasts. The most intense staining of TGFβ was found in calcified cartilage and mineralized bone matrix, again indicating preferential compartmentalization of TGFβ in the mineral phase. In contrast to the delayed expression of TGFβ protein, northern blot analysis showed TGFβ mRNA in implants throughout the sequence of bone formation. The time-dependent accumulation of TGFβ when cartilage is being replaced by bone in this in vivo model of bone formation suggests that TGFβ may play a role in the regulation of ossification during endochondral bone development.

TRANSFORMING growth factor β (TGFβ)[1] was initially characterized based on its ability to transform the phenotype of fibroblasts in vitro (Roberts et al., 1983). It is found in normal tissues and, although it is present in highest concentration in platelets (Assoian et al., 1983), bone represents the most abundant source of the peptide (Seyedin et al., 1985, 1986; Ellingsworth et al., 1986). TGFβ exists in two distinct homodimeric forms, of which TGFβ type 1 predominates (Cheifetz et al., 1987) but both TGFβ type 1 and TGFβ type 2 have been identified in bone (Seyedin et al., 1985). The presence of these peptides in bone suggests that they may play a role in bone formation or repair (Centrella and Canalis, 1985; Robey et al., 1987; Centrella et al., 1987). The fact that TGFβ is present in many tissues, cells, and in serum (O'Connor-McCourt and Wakefield, 1987), and that the receptor is also present on a wide spectrum of cell types (Wakefield et al., 1987), not only implies that it may play a basic role in cellular physiology, but also suggests that regulation of its effects is important. Therefore, systems in which TGFβ may function must be considered from the standpoint of regulation of the bioavailability of the factor and regulation of its activation from the latent form in which it is normally secreted (Miyazono et al., 1988; Wakefield et al., 1988).

Endochondral bone develops through a series of events including formation of cartilage, hypertrophy and calcification of the cartilage, vascular invasion, appearance of osteoblasts, and formation of bone. These events have been studied in embryonic development (Fell, 1925; Holder, 1978; Ede, 1983) and in a postnatal model of bone development (Reddi, 1981; Reddi, 1984). In both cases the sequence of events is remarkably similar. When implanted intramuscularly or subcutaneously in rats (Urist, 1965; Reddi and Huggins, 1972), demineralized bone matrix induces the formation of an ossicle of bone with bone marrow. The cellular processes of bone formation induced by implanted demineralized bone matrix are indistinguishable from embryonic bone forma-

1. *Abbreviations used in this paper*: EDTA-Gdn, EDTA and 4 M guanidine extraction buffer; Gdn, 4 M guanidine extraction buffer; TGFβ, transforming growth factor β.

tion. In addition, the bone induced remains for the life of the animal.

The advantages of using this system to study the role of TGFβ in bone development are severalfold. First, the process of matrix-induced bone formation in vivo is well characterized and recapitulates the embryonic developmental sequence. Currently it is impossible to investigate bone differentiation in vitro. In addition, while bone formation in developing fetal bone or growth plate is a continuum, there is only one cycle of bone formation in implants. Further, events take place in a more synchronous fashion throughout the tissue such that the predominant phases of bone formation can be distinguished using biochemical parameters (Reddi, 1981) and can be correlated with biological processes.

In the present study we used the matrix-induced bone forming system to examine the time course of appearance of TGFβ and its localization in developing endochondral bone. Proteins are easily extracted from bone by using 4 M guanidine buffers (Gdn) (Sampath and Reddi, 1984; Seyedin et al., 1985; Hauschka et al., 1986), and this extraction procedure was used to first isolate TGFβ 1 and TGFβ 2 from mature bone (Seyedin et al., 1985). We have used this procedure to extract, quantitate, and characterize the TGFβ in developing bone.

Materials and Methods

Quantitation of TGFβ Content in Developing Endochondral Bone

New bone formation was induced in 25–30-d-old male Long-Evans rats by implanting 30 mg of rat demineralized bone matrix subcutaneously on each side of the thoracic region as previously described (Reddi and Huggins, 1972). At selected days after implantation, the implants were recovered from four rats, yielding eight implants for each time point. One half of two implants from different rats at each time point were fixed immediately in Bouin's solution for 2 h, then transferred to 70% ethanol until they were embedded for immunohistochemistry. The remaining implants for each time point were pooled, minced, weighed, homogenized, and sequentially extracted in guanidine buffer then Gdn plus EDTA buffer (EDTA–Gdn). The first extraction was done overnight at 4°C using a filtered solution of 50 mM Tris-hydrochloride, pH 7.4, with 4 M guanidine hydrochloride, 100 mM 6-aminohexanoic acid, 5 mM benzamidine, 5 mM *N*-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% CHAPS. After centrifugation for 15 min at 4°C each supernatant and two washes of the pellet were pooled, extensively dialyzed against 0.5 M acetic acid, and lyophylized. The pellets were subsequently reextracted overnight with Gdn containing 0.5 M EDTA to demineralize and further extract the tissues. Extracted proteins from this step were processed as described for the first extraction. This two-step extraction procedure was designed to separate the proteins associated with nonmineralized matrix from those associated with mineralized matrix (Sampath and Reddi, 1984). Proteins which are strongly associated with the mineral component of bone matrix should be extractable only after dissolving the mineral with EDTA. Therefore, the sequential extraction with Gdn and then with EDTA–Gdn yields bone proteins which were not associated with mineral, then proteins which were bound to the mineral (Sampath and Reddi, 1984), respectively. As a control, nonmineralized tissue which had been similarly generated by implantation of non-bone-inductive Gdn-extracted matrix was extracted according to the same procedure. The previously Gdn-extracted demineralized bone matrix residue did not induce new bone formation when implanted as for active demineralized bone matrix. Instead, it was infiltrated by fibroblast-like cells and slowly resorbed. If separation of the mineralized and nonmineralized compartments was effective, we expected any TGFβ present in the nonmineralized control tissue to be extracted only in the first extraction.

To quantitate the amount of TGFβ at each time point, an aliquot of protein from each extraction method at each time point was dissolved in 4 mM HCl and analyzed in a competitive radioreceptor binding assay. A549 human lung carcinoma cells were plated in DME with 10% FCS. After 24 h, the cells were washed twice with 1 ml binding buffer (MEM, 25 mM Hepes, 0.1% BSA), and competitive binding of TGFβ to its cellular receptor was determined as previously described (Frolik et al., 1984). Four dilutions of each sample were added to ^{125}I-labeled TGFβ and the mixture was added to each well containing cells. After incubating at room temperature for 2 h, cells were washed four times with Hanks' balanced salt solution with 0.1% BSA at 0°C. Cells were solubilized in 1 M Hepes buffer with Triton X-100 and glycerol for 30 min at 37°C and triturated thoroughly. Radioactivity in aliquots was counted and competitive binding of samples was determined after subtracting nonspecific binding (determined in the presence of excess unlabeled TGFβ). The quantity of TGFβ was determined by comparison to a standard dilution curve for TGFβ (Robey et al., 1987).

Analysis of TGFβ 1 and TGFβ-2 Content of Developing Bone

An assay of TGFβ inhibition of CCL64 mink lung epithelial cell growth was used in combination with specific antibodies to TGFβ 1 and TGFβ 2 to determine the relative contributions of each form of the protein to the total TGFβ content of developing bone (Rosa et al., 1988). CCL64 cells were plated in wells of a 24-well plate at a density of 5×10^4 cells per well in 0.5 ml DME containing 0.2% FBS. After 1 h, bone extracts and antibodies were added to the wells as appropriate for the experiment and incubated for 22 h. At that time 0.5 μCi of ^{125}I-deoxyuridine was added to each well for 2 h. Cells were fixed, washed, and dissolved in 1 N NaOH, and incorporation of ^{125}I-deoxyuridine was counted as a measure of cell growth. The antisera used were turkey antiserum to TGFβ 1 and preimmune serum, rabbit IgG raised against TGFβ 2 and nonimmune rabbit IgG, and IgG purchased from R & D Systems, Inc. (Minneapolis, MN) raised against TGFβ 1 but which has cross-reactivity with TGFβ 2 (see Results).

DNA and Protein Determination

To determine DNA and protein content of each sample, a weighed amount of extract was dissolved in TCA and hydrolyzed at 90°C for 20 min, then quenched on ice. Samples were centrifuged to remove the insoluble protein and the supernatant was used for determination of DNA content using the diphenylamine method of Burton (1956). The protein pellet was dissolved in 0.1 N NaOH and heated at 37°C for 30 min. After centrifugation, the supernatant was then used for protein determination using a protein assay (Bio-Rad Laboratories, Richmond, CA). To determine the total DNA or protein at each time point, the amounts from Gdn extracts and EDTA–Gdn extracts were combined at each time point.

Immunodetection of TGFβ 1 on Nitrocellulose

Extracted protein from 12–21-d implants was dissolved in sample buffer and 210 μg of each extract was subjected to eletrophoresis in a 15% polyacrylamide gel, and then electrophoretically transferred to nitrocellulose. The membrane was incubated with turkey antiserum to TGFβ 1 in PBS containing 0.05% Tween 20. After washing and incubating with a biotinylated secondary antibody, and then peroxidase-conjugated avidin–biotin complex (Vector Laboratories, Inc., Burlingame, CA), the membrane was developed in 6 mg/ml 4-chloro-1-napthol in a PBS solution containing methanol and hydrogen peroxide.

Immunolocalization of TGFβ

Tissues that had been harvested and fixed as described above were embedded in paraffin and 5-μm sections were made according to standard procedures. After blocking endogenous peroxidase activity, sections were pretreated with hyaluronidase and nonspecific binding of antibody was blocked using 0.5% BSA and 1:20 normal goat serum in Tris-buffered saline. Sections were then incubated overnight with 5 μg/ml of either rabbit anti-TGFβ IgG or control IgG in blocking solution. The antiserum was anti-TGFβ 1 IgG prepared by protein A chromatography from rabbit antiserum raised against a peptide corresponding to the first 30 amino acids of TGFβ 1 (Heine et al., 1987). Control IgG was prepared by preadsorbing anti-TGFβ 1 IgG on a TGFβ 1-linked affinity column to eliminate TGFβ 1-specific antibody (Heine et al., 1987). Binding of antiserum to sections was visualized using biotinylated anti-rabbit secondary antibody (Cappel Laboratories, Cochranville, PA) followed by avidin–biotin complex reagent (Vector Laboratories, Inc., Burlingame, CA) and development with 0.05% 3, 3'-diaminobenzidine.

Analysis of RNA for TGFβ Message

A series of implants was done corresponding to the time points used to quantitate TGFβ. These implants were immediately frozen in liquid nitrogen after harvest, then processed for isolation of RNA according to Chirgwin et al. (1979). RNA was stored under liquid nitrogen. For northern blot analysis, RNA was quantitated using 260 nm OD and equal amounts of whole RNA at different time points were taken for electrophoresis. Electrophoresis of RNA was done in 1% agarose formaldehyde gels and the gels were stained with ethidium bromide and photographed. The RNA was then transferred to a gene screen (New England Nuclear, Boston, MA) membrane by capillary action. For comparison to RNA from normal tissues, the tibias and femurs of 45-d-old rats were dissected clean of adhering tissues and then cut into epiphysis and diaphysis. Marrow was cleaned from the bones and collected. RNA was prepared from all three tissues for hybridization to a TGFβ 1 probe as were implant tissues as described above.

RNA was hybridized to an M13 cDNA probe for TGFβ 1 as previously described (Robey et al., 1987). The probe begins at nucleotide 1,736 and ends at nucleotide 1,955 corresponding to amino acids 229–371 of the pre-pro TGFβ 1 protein. The cDNA probe was labeled by primer extension with ^{32}P and hybridized according to the method of Church and Gilbert (1984). Blots were washed using 1.5 mM sodium citrate and 15 mM sodium chloride with 0.1% SDS at 55°C and exposed to x-ray film for 4–7 d at −70°C. To quantitate the hybridization, x-rays were scanned using an ultroscan densitometer (model 2202; LKB Instruments, Inc., Gaithersburg, MD).

Results

Characterization of the TGFβ Content of Developing Endochondral Bone

Unlike soft tissues, bone has two major compartments: one is composed of cells and unmineralized matrix; the other is mineralized matrix. In this experiment the tissues were extracted first with Gdn alone to solubilize proteins from the unmineralized compartment, and then with EDTA–Gdn to demineralize the sample and solubilize proteins which were more tightly bound or associated with the mineralized compartment. The TGFβ which could be extracted from developing bone and control tissues using these procedures was quantitated at each time point in a competitive radioreceptor binding assay. The results showed that the TGFβ present in control, nonmineralized implants, was extracted using Gdn alone. TGFβ was present in 3–9-d Gdn extracts of control implants but gave atypical curves in the radioreceptor assay. A second assay of these extracts using growth inhibition of CCL64 cells showed that TGFβ was present at 0.5–1.0 ng/mg protein. TGFβ was undetectable in the second, EDTA–Gdn extracts of control tissues. Just the opposite was true in bone-forming implant extracts where TGFβ was not detected in Gdn extracts either by radioreceptor assay or immunoblotting (see below). Instead, the TGFβ in developing bone was solubilized only after demineralization and was detected in the EDTA–Gdn extracts. The radioreceptor assays of EDTA–Gdn extracts from bone-forming tissues yielded curves which were parallel to the standard curve for TGFβ (Fig. 1 *a*). The concentration of TGFβ was very low at day 1 and undetectable on days 3–7. Beginning on day 9 of bone formation, the concentration of TGFβ rose steadily through day 14, then remained high through day 21 (Fig. 1 *b*).

TGFβ was present at 8 ng/mg protein in extracts of adult rat bone matrix which had been acid demineralized under nondissociative conditions. This was the same matrix that was implanted to induce bone formation. As expected, due to the prior demineralization, the TGFβ was extracted from this matrix by Gdn alone. This also demonstrated that TGFβ was a normal component of adult rat bone matrix. TGFβ was not detected in extracts of demineralized bone matrix which had been previously extracted with Gdn (the same matrix implanted to generate control tissues for this assay).

Day 21 developing bone contained less TGFβ per mg extracted protein than the preparation of demineralized bone matrix from more mature bone. This suggests that TGFβ levels in bone may gradually increase, possibly accompanying remodeling processes after day 21. A similar situation is found for bone Gla protein, a vitamin K–dependent protein containing three residues of γ carboxyglutamic acid, which also has an affinity for the mineralized compartment and which is continually incorporated into bone matrix that has undergone several cycles of remodeling after day 21 (Price et al., 1981; Hauschka and Reddi, 1980).

To further characterize the TGFβ in bone-forming tissues, immunoblots were done using turkey antiserum to TGFβ 1. The results showed that the TGFβ activity identified by radioreceptor assay was also immunologically identifiable as TGFβ 1 and was found in EDTA–Gdn extracts but not Gdn extracts (Fig. 2).

Because both TGFβ 1 and TGFβ 2 have been found in mature bone (Seyedin et al, 1985) we examined the proportion of the two types of TGFβ found in 11- and 14-d developing bone extracts. Blocking antibodies specific to TGFβ 1 and 2 were used to neutralize the growth inhibitory activity of the extracted TGFβ on CCL64 cells. Curves generated using EDTA–Gdn extracts of bone-forming tissues were parallel to the standard curve (Fig. 3 *a*). In control experiments, either TGFβ 1 or TGFβ 2 was added to CCL64 cells in the presence of antibodies. As shown in Fig. 3 *b* , antibody to TGFβ 1 in-

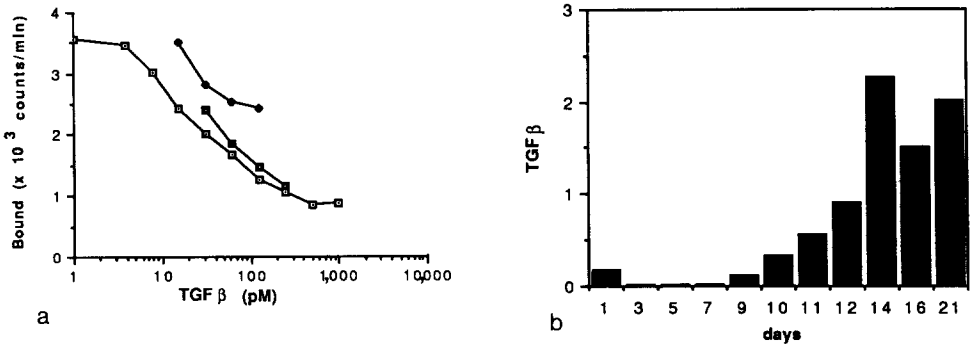

Figure 1. Results of assays of the ability of EDTA–Gdn extracts to compete with ^{125}I-labeled TGFβ 1 for binding to A549 cells. (*a*) TGFβ 1 standard curve (□) and curves for binding in the presence of extracts from day 11 (♦) and 14 (■) tissues. (*b*) Quantities of TGFβ detected at each time point given as ng TGFβ/mg extracted protein. Data shown are the average of two separate experiments at each time point.

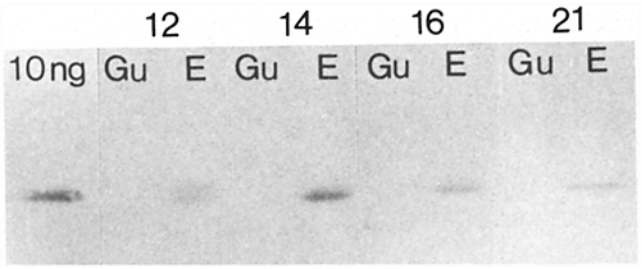

Figure 2. Immunodetection of TGFβ 1 in extracts of 12–21-d developing bone. The first lane contains 10 ng of TGFβ 1. *Gu*, lanes with Gdn extracts at each time point; *E*, lanes with EDTA–Gdn extracts.

hibited TGFβ 1 activity but not TGFβ 2 activity, whereas antibody to TGFβ 2 inhibited TGFβ 2 activity but not TGFβ 1 activity. In corresponding experiments on 11-d (calcified cartilage stage) extracts, 62% of the TGFβ activity was blocked by anti-TGFβ 1 antibody and 21% by anti-TGFβ 2 antibody (Fig. 3 *c*). In tests of 14-d extracts (bone formation stage), 72% of the TGFβ activity was blocked by anti-TGFβ 1 antibody and 18% by anti-TGFβ 2 antibody. Antibody from R & D Systems, Inc., which blocked both TGFβ 1 and 2 activity in controls, blocked 90% of the TGFβ activity in extracts at both time points.

Immunohistochemistry

Because TGFβ was present during bone formation and, therefore, could influence bone development, it was important to determine the site of localization. To examine the cellular and matrix localization of TGBβ in developing bone, sections of bone-forming implants were stained using 5 μg/ml anti-TGFβ 1 LC (1–30) IgG or, as controls, with IgG previously adsorbed to TGFβ; this antibody has recently been shown to stain intracellular sites of synthesis of TGFβ (Flanders, K. C., manuscript in preparation). On day 1, the cells in the interior of the bone-forming implant were mostly granulocytes, a subpopulation of which contained TGFβ (Fig. 4). TGFβ was not detected in the cells which formed a capsule around the implant or in cells just interior to the capsule. 3-d bone-forming implants had very few granulocytes which were stained for TGFβ. By day 7, TGFβ was detected in the small amount of calcifying cartilage in the bone-forming implants. In 11-d bone-forming implants, the matrix of calcified chondrocytes was heavily labeled for TGFβ. In addition, staining was seen in some osteoblasts adjacent to forming bone. At day 14 this distribution was similar with staining in osteoblasts, cartilage cells and calcified cartilage matrix (Fig. 5). On day 16 and 21, TGFβ was concentrated in newly formed bone matrix, remaining cartilage matrix, and osteoblasts. TGFβ was also detected in granulocytes in the forming bone marrow. Interestingly, with LC (1–30) antibody, no TGFβ was detected in osteocytes comparable to staining seen by Ellingsworth et al. (1986). Recent experiments have shown that these antibodies recognize different epitopes within the amino-terminal 1–30 amino acid sequence of TGFβ (Flanders, K. C., manuscript in preparation).

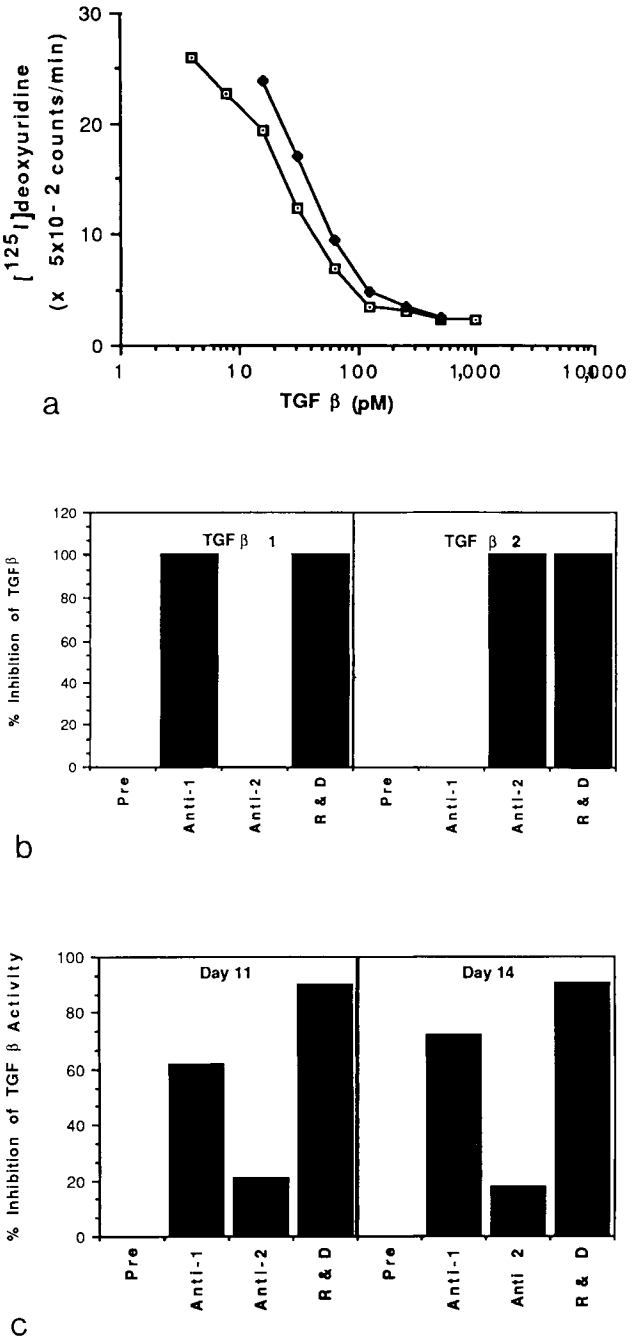

Figure 3. Results of inhibition of growth of CC164 cells by EDTA–Gdn extracts in the presence of antibodies to TGFβ 1 and 2. (*a*) Standard curve (⊡) and 11-d EDTA–Gdn extract curve (♦) showing growth inhibition of CC164 cells by TGFβ as measured by incorporation of ^{125}I-deoxyuridine. (*b*) Control experiments demonstrating specific inhibition of TGFβ 1 and 2 activity by corresponding antibodies. (*c*) Experiments using EDTA–Gdn extracts of 11–14-d bone-forming implants. *Pre*, preimmune serum; *Anti-1*, turkey antiserum to TGFβ 1; *Anti-2*, rabbit IgG to TGFβ 2; *R & D*, anti-TGFβ IgG from R & D Systems, Inc.

Detection of mRNA for TGFβ

The TGFβ detected in developing bone could result from endogenous production or from adsorption of serum TGFβ. Since immunohistochemical analysis indicated TGFβ was

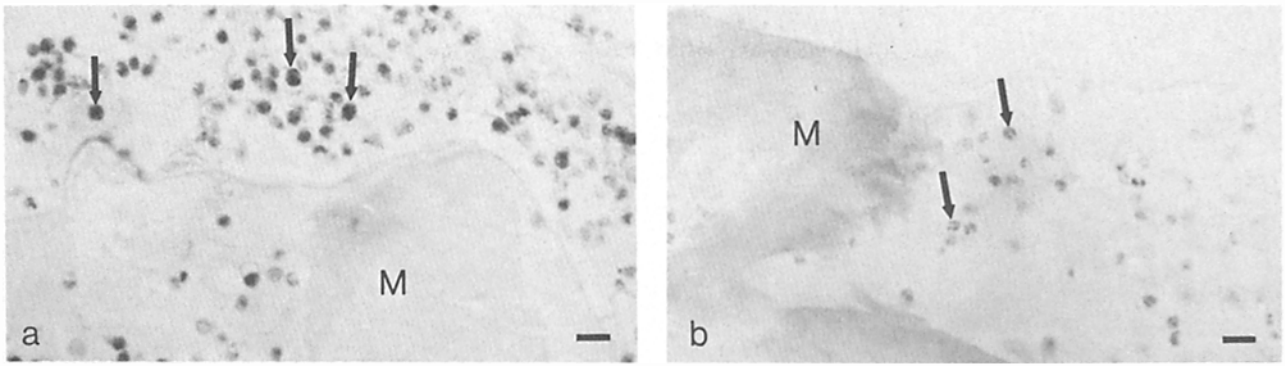

Figure 4. Sections of 1-d bone-forming implant stained with anti-TGFβ IgG (*a*) or control IgG (*b*). *M*, implanted matrix particles; *arrows*, polymorphonuclear leukocytes which stain specifically for TGFβ. Bar, 10 μm.

present in some cartilage cells and osteoblasts, we examined RNA prepared from implants for TGFβ 1 expression. Hybridization of RNA from developing bone implants with a cDNA probe for TGFβ 1 showed that a 2.5-kb transcript was present beginning at day 1 and continuing to day 21 with a decrease at day 21 (Fig. 6 *a*). We also examined 45-d-old rat epiphysis, diaphysis, and bone marrow for mRNA for TGFβ 1. mRNA for TGFβ was present in all three of these normal bone tissues including the epiphysis, where the growth plate had developmental processes similar to those we examined in developing bone implants (Fig. 6 *c*).

Discussion

In vitro studies have indicated that TGFβ may be important in bone development, remodeling, or repair (Robey et al., 1987; Pfeilschifter et al., 1987; Pfeilschifter and Mundy, 1987). In the present study, TGFβ was detected in developing endochondral bone in vivo. This is the first evidence that TGFβ is present during the process of bone formation, although it had previously been demonstrated to be in mature bone (Seyedin et al., 1985, 1986). TGFβ was present in highest concentrations at the time when the conversion from calcified cartilage to bone was taking place in the implants (see Fig. 7), and consequently when osteoblasts, known to both produce and be responsive to TGFβ in culture, were present.

TGFβ is found in many cells and tissues and the receptor is also widely distributed. Osteoblasts, in particular, have high affinity receptors for TGFβ (Robey et al., 1987). Therefore, in order for TGFβ to have regulatory effects, its activity must be precisely controlled within the tissue. The results of both extraction and immunohistochemical analysis of developing bone indicated that the TGFβ was tightly bound to mineralized matrix of calcified cartilage and bone. This compartmentation of TGFβ in the mineral phase may be a mechanism for storing latent or processed protein. Developing bone provides intrinsic possibilities for sequestration and accumulation of the protein during bone development and perhaps this will be a general biological mechanism for regulation of factors in bone. In order for the continuing process of endochondral bone formation to take place, the calcified cartilage matrix with its high concentration of TGFβ, must be broken down by chondroclasts. This is a process similar to that of bone remodeling, when existing bone matrix is broken down by osteoclasts in a sealed extracellular compartment which has acidic pH and lysosome-like characteristics (Baron et al., 1985). Both proteases and acid treatment are known to activate TGFβ from its inactive form (Lawrence et al., 1985; Miyazono et al., 1988). Experiments by Pfeil-

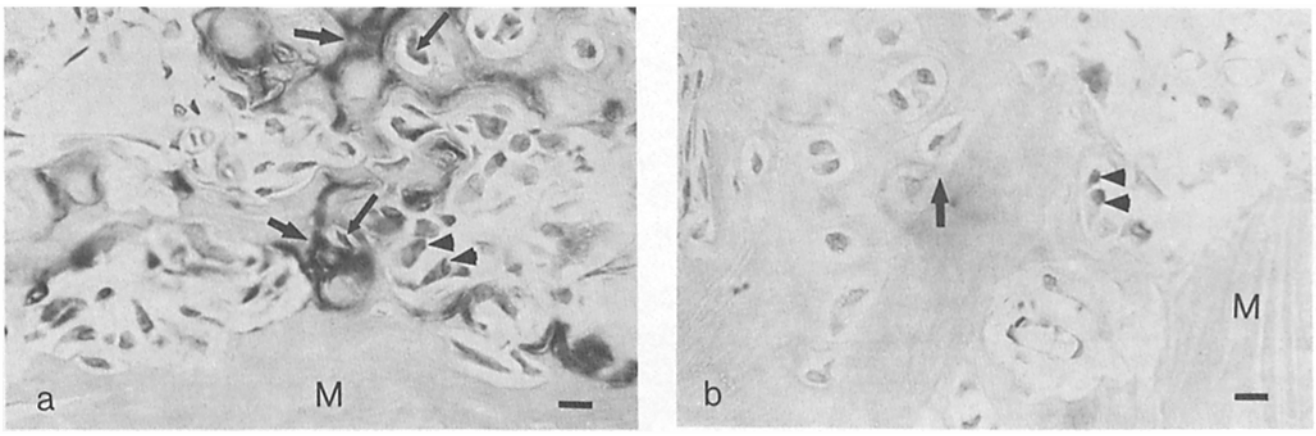

Figure 5. Sections of 14-d bone-forming implants stained with anti-TGFβ IgG (*a*) or control IgG (*b*). *M*, implanted matrix particles; *large arrows*, calcified cartilage matrix; *small arrows*, remaining calcified cartilage cells; *arrowheads*, osteoblasts. Bar, 10 μm.

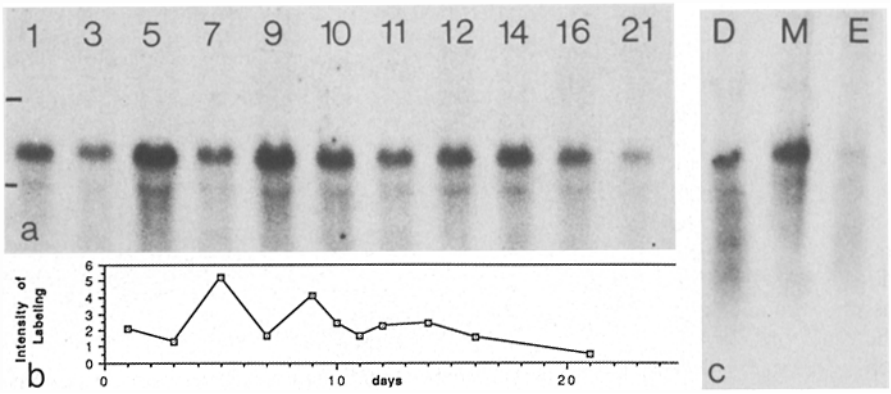

Figure 6. Photographs of autoradiograms from northern blots hybridized with a *C*-DNA probe for TGFβ 1. (*a*) RNA from bone-forming implants at the indicated days after implantation of demineralized bone matrix. *Bars*, the levels of 18 and 28 *s* ribosomal RNA bands. (*b*) Graph of densitometer readings of the autoradiogram in *a*. (*c*) RNA from 45-d-old rat diaphysis (*D*), bone marrow (*M*), and epiphysis (*E*).

schifter and Mundy (1987) indicate that TGFβ activity increases in cultures of resorbing calvaria, at least partially due to the resorbing of the bone itself. Therefore, the breakdown of cartilage matrix during ossification leads to the possibility of both release and activation of the TGFβ in the environment provided by osteoclasts and chondroclasts.

Assuming 1 g tissue is equivalent to 1 ml vol, wet weight tissue concentrations of TGFβ can be estimated from our results to have been between 2.5 and 49 ng/ml. The immunohistochemical analysis showed that the TGFβ was not evenly distributed but was concentrated in areas of calcified matrix, indicating that local concentrations of TGFβ are probably much higher than the above estimates. In vitro, osteoblasts and osteoblastic cell lines respond to TGFβ at concentrations as low as 0.5 ng/ml by either increasing or decreasing proliferation or alkaline phosphatase activity, depending on the density of the cells and the cell source used (Centrella et al., 1987; Robey et al., 1987; Elford et al., 1987; Pfeilschifter et al., 1987). Therefore, if even a small portion of the TGFβ sequestered in the calcified matrix were released and active, it could possibly affect surrounding osteoblasts. What effect TGFβ has on these cells in vivo is not clear since, depending on culture conditions and the cells used, various experimenters have reported differing effects (Centrella et al., 1987; Robey et al., 1987; Elford et al., 1987; Pfeilschifter et al., 1987).

In our experiments, both TGFβ 1 and 2 were present at approximately the same proportions at two different times during bone development and these proportions (~3:1 ratio of TGFβ 1 to TGFβ 2) are similar to the proportions of each form of the protein reported to be present in mature bone matrix (Seyedin et al., 1985). Since the amount of TGFβ increases fourfold between days 11 and 14 (see Fig. 1 *b*), our data suggest that TGFβ 1 and 2 are increased coordinately during the process of bone formation.

Intracellular immunohistochemical localization of TGFβ in calcifying cartilage and osteoblasts, and the demonstration of mRNA for TGFβ 1 in the forming bone implants suggests that the TGFβ extracted from the implants may have resulted from local production. The cell types in which TGFβ could be detected immunohistochemically varied with time after implantation: first inflammatory cells, then cells in late hypertrophying and calcifying cartilage, then osteoblasts and bone marrow granulocytes stained for TGFβ. However, since the increase in TGFβ content corresponded to the time of vascular invasion of the tissue, we can not exclude a contribution of TGFβ from serum.

Northern blot analysis indicated that the increasing

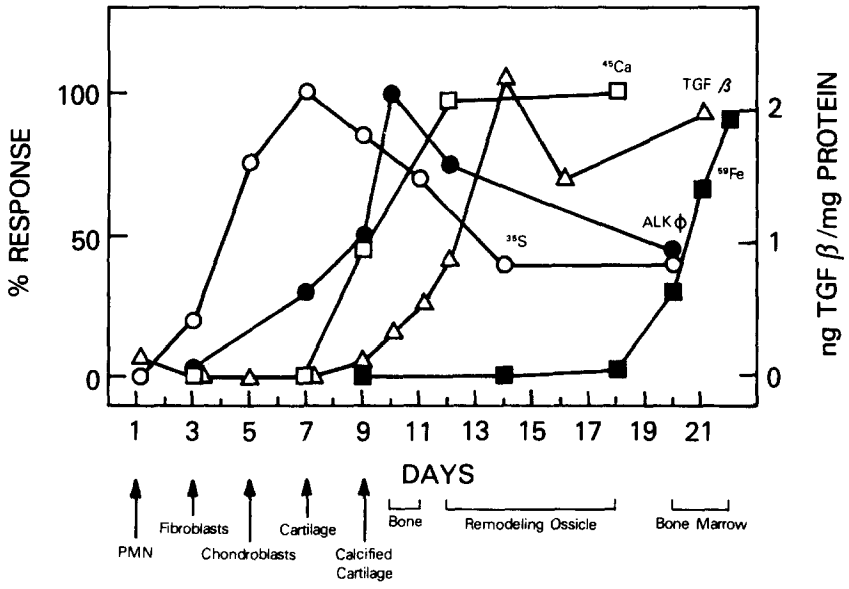

Figure 7. Quantities of TGFβ determined in this study in comparison to known parameters of endochondral bone formation in the matrix-induced bone system. Data for ^{35}S, ^{45}Ca, Alkaline phosphatase, and ^{59}Fe are from Reddi (1981). *PMN*, polymorphonuclear leukocytes. The increase in TGFβ content, as found in the present study, correlates with the time of increasing calcium incorporation and occurs at the time of appearance of calcified cartilage.

amount of TGFβ in the implants was not due to increased transcription. Similar situations are found in both activated lymphocytes (Kehrl et al., 1986) and macrophages (Assoian et al., 1987) where TGFβ mRNA levels remain constant while protein secretion increases, suggesting translational rather than transcriptional control. This may be the case in the present study as well. However, because the TGFβ appears to have a high affinity for the mineralized matrix in developing bone, the increase in protein content is likely to be the result of accumulation of secreted protein after synthesis.

Both TGFβ 1 and 2 were present during endochondral bone development when the critical transition from calcified cartilage to bone was taking place and when osteoblasts, cells which can be affected by TGFβ and which lay down the bone matrix during this transition, were present. We do not yet know what effects the presence of TGFβ may have on ossification. In addition, whether TGFβ 1 or 2 is more important in regulation of bone formation remains to be tested. Because of the possible intrinsic regulation of TGFβ concentration and accessibility in bone, its effects may differ at different times. However, the appearance of TGFβ at the time of ossification indicates it may be an important regulator of bone development in vivo as well as of cell function in vitro.

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