Osteoblasts Synthesize and Respond to Transforming Growth Factor-Type β (TGF-β) In Vitro

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Abstract. Transforming growth factor-type β (TGF-β) has been identified as a constituent of bone matrix (Seyedin, S. M., A. Y. Thompson, H. Bentz, D. M. Rosen, J. M. McPheron, A. Conti, N. R. Siegel, G. R. Gallup, and K. A. Piez, 1986, J. Biol. Chem. 261:5693–5695). We used both developing bone and bone-forming cells in vitro to demonstrate the cellular origin of this peptide. TGF-β mRNA was detected by Northern analysis in both developing bone tissue and fetal bovine bone-forming cells using human cDNA probes. TGF-β was shown to be synthesized and secreted by metabolically labeled bone cell cultures by immunoprecipitation from the medium. Further, TGF-β activity was demonstrated in conditioned media from these cultures by competitive radioreceptor and growth promotion assays. Fetal bovine bone cells (FBBC) were found to have relatively few TGF-β receptors (5,800/cell) with an extremely low K_d of 2.2 pM (high binding affinity). In contrast to its inhibitory effects on the growth of many cell types including osteosarcoma cell lines, TGF-β stimulated the growth of subconfluent cultures of FBBC; it had little effect on the production of collagen by these cells. We conclude that bone-forming cells are a source for the TGF-β that is found in bone, and that these cells may be modulated by this factor in an autocrine fashion.

Materials and Methods

Cell Culture

Primary cultures of FBBC were prepared as previously described (13) by incubating collagenase-treated subperiosteal bone fragments in low Ca ++ nutrient medium until the cultures were confluent. Cells were then passaged by trypsin-EDTA, and plated at varying densities for the experiments described below. Cells were maintained in medium with normal levels of Ca ++ for 48–72 h before the beginning of each experiment. Rat osteosarcoma cell lines, ROS 17/2 (22) and UMR 106 (26), were also used for comparison with the nontransformed FBBC.

Analysis of TGF-β mRNA

Various developing tissues dissected from fetal rats (day 19) and calves (3–5 mo of gestation), as well as from the Swarm rat chondrosarcoma were used for RNA extraction as previously described (6). RNA was also isolated from two rat osteosarcoma cell lines (ROS 17/2 and UMR 106), primary cultures of FBBC (42), cell cultures derived from bovine skin, tendon, articular cartilage, and smooth muscle, and from human lymphocytes (as a positive control).
Northern analysis was performed using 1.2% agarose formaldehyde gels followed by transfer of RNA onto nitrocellulose (21). Either a nick-translated insert (1,060-bp Eco RI fragment) of a human TGF-β cDNA probe (7), kindly provided by Dr. Rik Derynick (Genentech, South San Francisco, CA) or a single stranded (219 bp) subclone of that probe was radiolabeled and hybridized (Fig. 1a). The nitrocellulose blots were washed under standard stringent hybridization conditions (21).

**Biosynthesis of TGF-β by FBBC**

First passage FBBC were plated at a density of 20,000 cells/cm² and allowed to recover for 48-72 h. The cells were then incubated with 300 μCi of [35S]cysteine for 20 h in DMEM containing 2% dialyzed calf serum and 10% of the usual concentration of methionine and cysteine. After incubation, the medium was saved, the cells were washed several times with PBS, and subsequently extracted with acid-ethanol. The extracts were sonicated, centrifuged to remove insoluble debris, and the resulting supernatants were used for immunoprecipitation. Aliquots of media and acid-ethanol extracts, containing 5 × 10⁶ TCA-precipitable counts, were lyophilized and resuspended in 100 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 0.005% methiolate) and boiled for 4 min. The samples were precipitated three times with normal rabbit serum (20 μl) and washed Staphylococcus aureus (immunoprecipitin from Bethesda Research Laboratories, Gaithersburg, MD) (20 μl of pelleted S. aureus resuspended in 100 μl of immunoprecipitation buffer). An IgG fraction of rabbit anti-human TGF-β (34), prepared by 50% ammonium sulfate precipitation, was added to the supernatant from the third preprecipitation, and the samples incubated overnight at 4°C. In some cases, the antibody was incubated overnight at 4°C with human platelet TGF-β before being added to the metabolically labeled sample in order to determine the specificity of the immunoprecipitation. The immunoreactive TGF-β was recovered by incubation with 100 μl equivalent S. aureus for 45 min at room temperature. After centrifugation the pellet was washed three times with immunoprecipitation buffer. The washed pellet was boiled for 4 min in a buffer containing 2% SDS and the supernatant obtained after centrifugation was subjected to electrophoresis on a 10% polyacrylamide gel according to Laemmli (17). In some cases, samples were boiled in the presence of 5% 2-mercaptoethanol and subjected to electrophoresis on 12.5% polyacrylamide gels. The gels were fixed, enhanced with 2.5 diphenylloxazole dissolved in DMSO, dried, and exposed to Kodak XAR-5 X-ray film at ~70°C (4).

**Secretion of TGF-β by FBBC**

Cells were plated at varying densities and allowed to recover for 48 h. In addition, wells containing no cells were set up and incubated identically for the generation of control (no cell) medium to account for the possible release of serum during the subsequent incubations. The cells were then washed once with serum-free medium, and incubated in serum-free DMEM containing 50 μg/ml ascorbic acid, glutamine, and penicillin/streptomycin. After 48 h, the medium was removed and the cell number determined. The conditioned media samples (either tested directly or after acid activation (18, 19)) were assayed for TGF-β activity both by a competitive radioreceptor assay (simultaneous addition of competitor and radiolabeled TGF-β) using A549 cells (11) and by a bioassay that measures the ability of the peptide to induce normal rat kidney fibroblasts (NRK) colony formation in soft agar in the presence of epidermal growth factor (31). Human platelet TGF-β (2) was used as a standard in these assays. In both assays, the original concent-

![Figure 1. Northern blot analysis of TGF-β mRNA from rat and bovine tissues and cultured cells. (a) A human TGF-β cDNA was radiolabeled by hybridization of synthetic oligonucleotides complementary to the 3' end of the cDNA followed by polymer extension using 32P-nucleotides and DNA polymerase, or by electrolution of insert cDNA followed by nick translation with α-32P-dCTP. Both probes contained the sequence encoding for the expressed amino acid sequence of TGF-β. The λβC probe extends from nucleotide 996 to nucleotide 1,051 (amino acid 53 to beyond the end of the TGF-β molecule). The M13 probe begins at nucleotide 1,736 and ends at nucleotide 1,955 (aa 229-371). (b) RNA was isolated from fetal rat tissues (calvarial, epiphyseal, and diaphyseal bone, skin, and muscle), the Swarm rat chondrosarcoma, and from bovine tissues (ameloblastic layer of developing teeth, cornet, liver, and brain). RNA from cultures of two rat osteoblastic cell lines (ROS 17/2 and UMR 106), primary FBBC taken from fetuses of 3 and 5 mo, and fetal bovine tendon,
Scatchard Analysis of TGF-β Binding

Cultures of FBBC as well as ROS 17/2 and UMR 106 rat osteosarcoma cell lines were plated at ~70-95% confluency. After 24 h for recovery, cells were preincubated in serum-free medium for 2 h at 37°C to eliminate endogenously bound ligand, and used for the determination of receptor number/cell and the dissociation constant (Kd) by Scatchard analysis using 125I-labeled human platelet TGF-β as previously described (II).

Biological Effects of TGF-β

FBBC were plated at various densities and allowed to recover for 24 h in DME containing 10% FBS, at which time the plating efficiency was determined. The cells were washed once with serum-free medium, and subsequently treated with purified human platelet TGF-β (0–40 pM/ml) in DME containing 2% FBS. The effects of TGF-β on the growth of cells were assessed after 48 h (FBBC) or after 144 h (ROS 17/2 and UMR 106) by releasing 100% of the cells (as determined by microscopic evaluation) with trypsin-EDTA, and direct cell count using a Coulter counter.

The percentage of collagen synthesized by the cells in the absence and presence of TGF-β (10 and 50 ng/ml) after 18 h of treatment was determined by labeling the cells with [3H]proline (20 µCi/ml) for 3 h as described (34) and analyzing both the medium and cell layer using the collagenase digestion method of Peterkofsky (28).

Results

Distribution of TGF-β mRNA in Tissues and Cultured Cells

The relative distribution of TGF-β mRNA in developing rat and bovine tissues, and in a variety of cell cultures was investigated by Northern analysis of extracted RNA using human TGF-β cDNA probes (Fig. 1 a). In fetal rat tissues, substantial amounts of hybridization were detected to a message of Mr 12,500 after reduction of disulfide bonds, identical to the peptide of Mr 25,000 that was converted to a polypeptide of Mr 12,500 after reduction of disulfide bonds, identical to the behavior of 125I-labeled human platelet TGF-β (Fig. 2). The immunoprecipitation of these polypeptides was blocked by the pretreatment of the antibody with pure, unlabeled TGF-β. Interestingly, relatively little TGF-β was identified in the cell layer fractions, suggesting that the peptide is secreted from, but not bound in the cell layer at the density used in this experiment, or in the absence of mineralization. A long radio-labeling time (20 h) was used in this experiment to assure that sufficient radiolabeled TGF-β accumulated in the medium to be detected by immunoprecipitation. The experimental design reduced the probability that a significant amount of TGF-β would be detected in the cell layer. However, studies with Ha-ras-transfected NIH-3T3 cells radiolabeled under a variety of conditions indicate that TGF-β is rapidly secreted from the cells making the intracellular TGF-β content below the detection limit (Flanders, K. C., unpublished results). Further, [35S]cysteine-labeled TGF-β could only be precipitated from the medium (that was not acid activated) after boiling the sample, while 125I-labeled human platelet TGF-β (which was previously acid activated) can be precipitated without boiling. This observation indicates that boiling either unmasks epitopes recognized by the antibody, or is equivalent to acid activation in the conversion of latent...
TGF-β to the active form (Flanders, K. C., unpublished results).

The biological activity of the TGF-β in the conditioned medium of FBBC was assayed both by competitive radioreceptor binding (Fig. 3) and by stimulation of NRK cell colony formation in soft agar in the presence of epidermal growth factor (not shown). The concentration of TGF-β present in the acid-activated conditioned medium of cultures was assayed both by competitive radioreceptor binding assay using A549 cells and human platelet TGF-β as a standard (11). The concentration of TGF-β in 48-h medium conditioned by FBBC cells at varying densities was determined in the binding assay and plotted versus the log of the cell number. Monolayer confluency was reached at a log cell number of 5.7. Control (no cell) medium and unconditioned medium had no assayable TGF-β. Data represents the average of duplicate determinations for one of two representative experiments.

Characterization of TGF-β Receptor Binding

The binding of [125I]-labeled human platelet TGF-β to receptors on FBBC was compared with that on the rat osteosarcoma cell lines (ROS 17/2 and UMR 106) by Scatchard analysis (Fig. 4). All three cell types had relatively few receptors per cell (2,200 ± 550 for ROS, 7,100 ± 3,300 for UMR, and 5,600 ± 160 for FBBC) with very low K_d values of 3.0 ± 0.4, 7.8 ± 1.9, and 2.0 ± 0.2 pM, respectively. Analysis of the binding of TGF-β to the FBBC, but not the cloned osteosarcoma cell lines, suggested the existence of a second class of receptors of a higher abundance (10,000/cell) and relatively higher K_d of 12 pM.

Modulation of Osteoblast Activity in TGF-β

The addition of low levels (<40 pM) of human platelet TGF-β to FBBC plated at varying densities stimulated cell growth (Fig. 5A). Two days after addition, 24-40 pM TGF-β caused a twofold increase in the number of cells plated at low density (10,000 cells/cm²). Interestingly, at both sparse and high cell densities, TGF-β had little mitogenic effect. The growth of both ROS 17/2 (Fig. 5B) and UMR 106 (not shown) osteosarcoma cells at all densities studied was inhibited by TGF-β at the same concentrations that stimulated growth of first passage FBBC cells. Again, the inhibitory effect was less pronounced when the cells were plated at higher densities.

Since TGF-β has been shown to significantly increase synthesis of collagen and fibronectin by fibroblastic cells, its ability to modulate synthesis of matrix proteins by FBBC was also examined. TGF-β (10 and 50 ng/ml) had an overall stimulatory effect (1.5-fold) on protein synthesis in FBBC.
However, there was no effect on the percentage of bacterial collagenase-sensitive protein secreted by the cells, which was found to be 50% in control, and 45 and 47% in cultures treated with 10 and 50 ng/ml, respectively. Analysis of the collagenase-sensitive protein secreted by the cells, which was found to be 50% in control, and 45 and 47% in cultures treated with 10 and 50 ng/ml, respectively. Analysis of the cell layers fractions were similar. In contrast, TGF-β increased relative collagen secretion in ROS 17/2 cells from 1.4 (control) to 3.8% (50 ng/ml TGF-β treatment). While this is a threefold increase, it should be noted that the overall percentage of collagen secreted by these cells was substantially less than that of FBBC (1.4 as compared with 50%).

**Discussion**

The recently established identity of TGF-β and cartilage-inducing factor A (36) and the recently reported immunolocalization of TGF-β to bone (8), indicates that bone is perhaps the most abundant source of TGF-β in the body (14, 35), with a concentration ~100-fold greater than that in soft tissues such as placenta (10) or kidney (32). We show here that one of the sources of TGF-β in bone is the bone-forming cells themselves. These cells were found to (a) transcribe TGF-β mRNA; (b) synthesize and secrete this peptide; (c) bear high affinity cell surface receptors, and (d) be mitogenically stimulated by TGF-β. The amount of TGF-β secreted by the confluent FBBC is ~sixfold higher than from virally transformed cells and between 10- and 20-fold higher than from nontransformed cells (1). Although the TGF-β secreted by FBBC in vitro is predominantly in a latent form that must be activated before it can be recognized by antibodies or can bind to its receptor, up to 25% is in the active form. The implications of this for a potential autocrine action of TGF-β on FBBC are not presently understood.

The dissociation constants (Kd) for binding of TGF-β to receptors on either FBBC or the rat osteosarcoma cell lines, ROS 17/2 and UMR 106, are very low (2-6 pM), indicating the presence of very high affinity-binding sites on these cells. Scatchard analyses of the binding of TGF-β to 33 different cell lines have shown that cells of mesenchymal origin have 7,000–80,000 receptors/cell with dissociation constants ranging from 13 to 60 pM (41a); these osteoblastic cells are thus distinguished by their low receptor number and high binding affinity. A second, more abundant, class of receptors with a relatively lower affinity was also identified (Fig. 4 c). These receptors are not of the very low affinity class (Kd of 4 nm) previously described (11), but are similar to those found in bovine skin fibroblasts derived from fetuses of the same gestational age (Roberts, A. B., and P. Gehron Robey, unpublished results). Thus, they may be indicative of either the presence of undifferentiated osteoblasts or fibroblasts present in small numbers in the culture. Alternatively, this second class of receptors may account for the binding of TGF-β to a receptor for a closely related molecule, cartilage-inducing factor B (35) with a relatively lower affinity for TGF-β.

In this system, TGF-β appears to promote the growth of the same cells that produce it. Moreover, the finding that ~25% of the secreted TGF-β is in an active form suggests that it might potentially function as an autocrine growth factor for these cells. However, it is possible that the mitogenic effect of TGF-β may be the consequence of the induction of other growth factors by the cells as has been shown in the AKR-2B cell line (37, 20). The stimulation of the anchorage-dependent growth of FBBC by TGF-β is in striking contrast, however, to its inhibition of anchorage-dependent growth of the two osteosarcoma cell lines studied here, as well as that of many other cell types including a clonal murine calvaria-derived cell line, MC3T3E1 (25), fibroblasts (33, 41), epithelial cells (23, 33), myoblasts (9), hepatocytes (15, 24), and endothelial cells (3); before this, only primary human mesothelial cells have been reported to be stimulated to grow in monolayer culture by TGF-β (12). The density-dependent effects of TGF-β on stimulation of growth of FBBC are analogous to those seen on inhibition of growth of either fibroblasts (32) or the osteosarcoma cells (Fig. 5 B) by this peptide (i.e., the growth of subconfluent cultures of ROS 17/2 cells or of NRK fibroblasts is inhibited by TGF-β, but the effect is diminished as the cells approach confluency).

The different biological response patterns to TGF-β of the two rat osteosarcoma cell lines compared with FBBC emphasize that the osteosarcoma cell lines may not exhibit all of the characteristics of the osteoblastic phenotype as had been previously suggested, based on their response to parathyroid hormone (22, 27). Thus, identical concentrations of TGF-β inhibit the growth of the osteosarcoma cell lines and stimulate the growth of FBBC. Moreover, ROS 17/2 cells but
not FBBC, respond to TGF-β with a selective increase in collagen synthesis compared with that of noncollagenous proteins. Significant differences were found, however, in the protein synthetic pattern of these two cell types (collagen secretion relative to total secreted proteins was 1.4 and 50% for ROS 17/2 and FBBC, respectively). Also in contrast to FBBC, fibroblastic cells are stimulated to increase collagen synthesis by TGF-β (16, 34). These data taken together suggest that TGF-β may serve as a mitogen for FBBC to expand this population of cells rather than to induce them to elaborate more matrix (i.e., become more differentiated). Expansion of this population of cells would also result in the elaboration of more TGF-β which could, in turn, act on other target cells. However, it should be noted that the effect of TGF-β on collagen production by FBBC cells may be density dependent, and further studies are needed to determine the effect of TGF-β on protein synthesis in superconfluent FBBC when matrix production and mineralization are initiated.

The abundance of TGF-β in bone (14, 35), the high affinity receptor binding, and the stimulation of the growth of FBBC, taken together with the finding that TGF-β can induce secretion of collagen and cartilage proteoglycans in mesenchymal cells (16, 34, 35) suggest a unique role for TGF-β in the growth and remodeling processes of hard tissues. Moreover, the findings that TGF-β stimulates bone resorption (39) and is itself released by organ cultures of developing bone exposed to parathyroid hormone (29) suggest that TGF-β may be involved in the "coupling" of bone resorption and bone formation to maintain normal rates of bone turnover (29). Further studies may clarify its role in bone metabolism in normal and diseased states.

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