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Recombinant Transforming Growth Factor Type β3: Biological Activities and Receptor-Binding Properties in Isolated Bone Cells

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We have recently cloned the cDNA for transforming growth factor type β3 (TGF-β3), a new member of the TGF-β gene family. We examined the biological effects of recombinant TGF-β3 protein in osteoblast-enriched bone cell cultures. In this report we demonstrate that TGF-β3 is a potent regulator of functions associated with bone formation, i.e., mitogenesis, collagen synthesis, and alkaline phosphatase activity. In a direct comparison between TGF-β3 and TGF-β1, TGF-β3 appeared to be three- to fivefold more potent than TGF-β1. Our cross-linking experiments with iodinated TGF-β showed that in osteoblast-enriched bone cell cultures, both TGF-β3 and TGF-β1 associated with the same three cell surface binding sites. Scatchard analysis of receptor competition studies indicated the presence of high-affinity binding sites for TGF-β3 in the picomolar range. TGF-β3 showed an approximately fourfold-higher apparent affinity than TGF-β1 in overall binding.

Type β transforming growth factors (TGF-βs) are multifunctional growth modulators which play a central role in embryonic development, tissue repair, immunoregulation, fibrosis, and carcinogenesis (18, 26). The two well-described 25-kilodalton (kDa) homodimer forms, TGF-β1 and TGF-β2, share 71% amino acid sequence identity. A heterodimer, composed of one polypeptide chain each from TGF-β1 and TGF-β2, termed TGF-β1.2, has been found in porcine platelets (6). In most assay systems, TGF-β1 and TGF-β2 have similar biological properties, although dramatic differences have also been observed. TGF-β1 has been claimed to be a 100-fold more potent growth inhibitor of hematopoietic stem cells than TGF-β2 (20). In addition, TGF-β2 but not TGF-β1 has been reported to induce mesoderm formation in early frog embryos (23). Differences in tissue distribution and gene regulation also suggest distinct biological roles for various TGF-β isoforms (26).

Recently, we identified another human form of TGF-β, termed TGF-β3. The mature bioactive form of TGF-β3 shares 80% amino acid sequence identity with TGF-β1 and 83% sequence identity with TGF-β2 (28). TGF-β3 mRNA expression in human cells has been found in umbilical cord and in a number of mesenchymal and tumor cell lines (9, 28). We have now expressed recombinant TGF-β3 and purified this protein to homogeneity by using an immunoaffinity column (28a).

TGF-β-like activities are produced by bone cells, and large amounts are found in the extracellular bone matrix, suggesting an important physiological function of TGF-βs in this tissue (4). TGF-β stimulates cell replication and collagen production in cultured fetal rat bone cells (2–5) and induces chondrogenesis of embryonic rat mesenchymal cells (25). In addition, molecules with TGF-β-like activity are released in vitro after bone resorption and may effect a link between the coupled processes of bone formation and resorption during remodeling (22, 27).

The experimental paradigm we have used in this report is to isolate various cell populations from resected bone fragments by sequential collagenase digestions (2–5). The later-released populations are enriched for bone-forming cells with the biochemical characteristics associated with the osteoblast phenotype, such as type I collagen production, elevated alkaline phosphatase activity, and osteocalcin synthesis (17). Studies with such isolated bone cells have shown that TGF-β1 is a potent regulator of cells from the osteoblast lineage (3). On a molar basis, TGF-β1 is one of the most potent mitogens thus far described for osteoblast-enriched cultures from fetal bone. The mitotic response to TGF-β1 is biphasic, with an optimal concentration below 100 pM (2–4). TGF-βs, in addition, alter expression of various activities associated with the osteoblast phenotype: alkaline phosphatase activity is decreased, while the synthesis of type I collagen is enhanced, similar to the effects of TGF-β in a number of other connective tissue systems (2, 3).

TGF-β1 and -β2 appear to act via binding to specific cell membrane receptors linked to as yet unknown intracellular signaling pathways. Virtually all cell types examined to date carry TGF-β receptors, with the exception of retinoblastoma and pheochromocytoma cells (14). The binding of TGF-β1 and TGF-β2 to integral cell membrane receptors has been demonstrated by chemical cross-linking. Three size classes of ligand-receptor complexes have been identified: (i) a 65-kDa glycoprotein; (ii) an 85- to 110-kDa glycoprotein; and (iii) a heterogeneous proteoglycan with an average size of 250 kDa (2, 7, 16, 24, 29). Current evidence suggests that the type I TGF-β receptor is required for TGF-β1's effect on DNA, collagen, and fibronectin synthesis (1, 24).

The present studies were performed to assess the effects of human recombinant TGF-β3 on osteoblast-enriched cultures from fetal rat parietal bone and to characterize the specific binding of TGF-β3 to bone cell surface proteins.

MATERIALS AND METHODS

Test agents. Recombinant human TGF-β3 protein used in these studies was obtained from conditioned medium of Chinese hamster ovary (CHO) cells transfected with an expression plasmid containing the TGF-β3 cDNA. The TGF-β3 protein was purified to homogeneity as judged by

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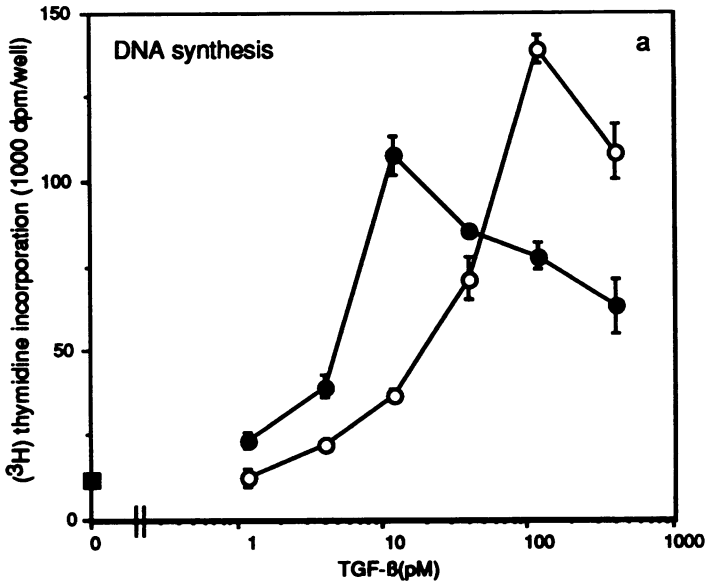
DNA synthesis
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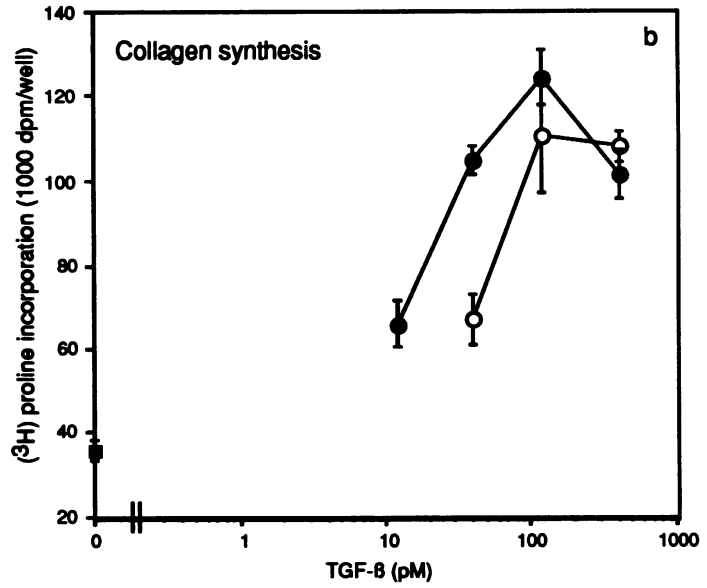
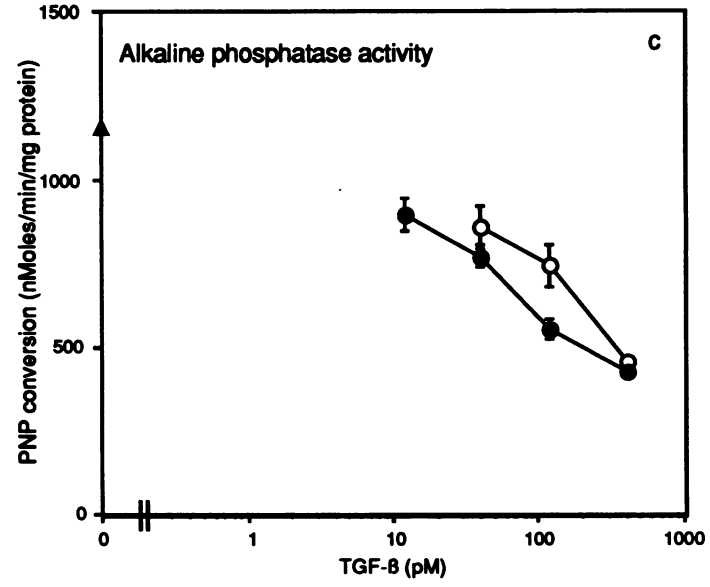
150
100
50
0
0
1
10
100
1000
TGF-ß(pM)


Collagen synthesis
b
(3H) proline incorporation (1000 dpm/well)
140
120
100
80
60
40
20
0
1
10
100
1000
TGF-ß (pM)


Alkaline phosphatase activity
c
PNP conversion (nMoles/min/mg protein)
1500
1000
500
0
0
1
10
100
1000
TGF-ß (pM)

silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28a). Human TGF-β1, purified from platelets, was obtained from Calbiochem (La Jolla, Calif.). TGF-β1 (from Biomedical Technologies, Stoughton, Mass.) and TGF-β3 were iodinated on ice for 15 s by a modification of the method of Hunter and Greenwood (11). The radiolabeled TGF-β was separated from the free iodine by adsorption to a Waters SEP-Pak C18 cartridge, which was pretreated with bovine serum albumin. The iodinated TGF-β was eluted with 80% acetonitrile–0.05% trifluoracetic acid.

Cell cultures. Parietal bones dissected free of adjacent suture lines were obtained from 22-day-old rat fetuses (Sprague-Dawley; Charles River Breeding Laboratories, Wilmington, Mass.) and subjected to five sequential 20-min collagenase digestions as described previously (17, 30). The population of cells released during the first enzyme treatment (population 1) is enriched with less-differentiated fibroblastlike cells, whereas the last three (populations 3 to 5) are enriched with cells expressing features characteristic of the osteoblast phenotype. Cells from population 1 and a pool of cells from populations 3 to 5 were plated at 12,500 cells per cm^2 and cultured in Dulbecco modified Eagle medium as detailed previously (2, 3, 17). After reaching confluence (approximately 6×10^4 cells per cm^2), the cultures were deprived of serum for 20 h; the factors of interest were then added to the cultures in serum-free medium and incubated for an additional 23 h.

DNA synthesis. To examine the mitogenic effect of the test factors, cell cultures were pulse labeled with [^{3}H]thymidine (80 Ci/mmol) for the last 2 h of treatment and lysed by the addition of 0.1% sodium dodecyl sulfate and 0.1 N sodium hydroxide. The insoluble material formed by precipitation with 10% trichloroacetic acid was collected on glass fiber filters, rinsed with ethanol, and measured by scintillation counting. Data are shown as the total amount of acid-precipitable [^{3}H]thymidine incorporated per 0.32-cm^2 culture well.

Protein synthesis. To measure collagen and noncollagen protein synthesis, 2-cm^2 cultures were pulsed with 12.5 μCi of [^{3}H]proline (125 mCi/mmol) per ml for the last 2 h of culture. Cells were rinsed with isotonic buffer (146 mM NaCl, 11 mM dextrose, 35 mM Tris hydrochloride [pH 7.4]) and lysed by freeze-thawing in 0.5% (vol/vol) Triton X-100 (Sigma Chemical Co.). The homogenates were diluted threefold and precipitated with 10% trichloroacetic acid, and the acid-precipitable material was collected by centrifugation. The pellets were acetone extracted, dried, resolubilized in 0.5 M acetic acid, and neutralized with NaOH. The amount of [^{3}H]proline incorporated into collagenase-digestible protein and noncollagen protein was measured as described by Peterkofsky and Diegelmann (21).

Alkaline phosphatase assay. Enzyme activity was measured in extracts prepared from 2-cm^2 cultures following sonication in 0.5% Triton X-100. Hydrolysis of *p*-nitrophenyl phosphate was measured at 410 nm after 30 min (15); data are expressed as nanomoles of *p*-nitrophenol released per minute per milligram of protein.

Receptor assays. Binding of TGF-β1 and TGF-β3 to cell surface-associated binding sites was examined by methods adapted from Massague and Like (16). Confluent cell cultures (4.8 cm^2) were serum deprived for 20 h, rinsed, incubated for 1 h at 37°C, rinsed, and incubated for an additional hour at 4°C in serum-free medium containing 0.4% bovine serum albumin. ^{125}I-labeled TGF-β (80 pM) in the presence or absence of unlabeled TGF-β1 or -β3 was then added for 3 h at 4°C; the cultures were rinsed with cold isotonic buffer and cross-linked by a 15-min incubation at 4°C with 0.5 mM disuccinimidyl suberate. Subsequently, cells were extracted, and the amount of bound ligand was determined in a gamma counter and electrophoresed on 7.5% polyacrylamide gels. Bound [^{125}I]TGF-β was visualized by autoradiography as reported previously (2).

Statistical methods. Data for effects on DNA and collagen synthesis and alkaline phosphatase activity are shown as the means ± standard error of the mean (SEM) and are representative of at least three independent determinations. Binding studies were performed with at least two separate batches of [^{125}I]TGF-β1 or [^{125}I]TGF-β3 and are representative of at least two independent determinations.

RESULTS

Effects of recombinant TGF-β3 on DNA synthesis rates, collagen production, and alkaline phosphatase activity. We examined the effects of recombinant human TGF-β3 and human platelet-derived TGF-β1 in osteoblast-enriched cultures obtained from fetal rat parietal bone (Fig. 1). The protein concentrations of TGF-β3 and TGF-β1 were normalized by using both the colloidal gold assay (Collaborative Research, Bedford, Mass.) and intensity of silver staining on an SDS-polyacrylamide gel. Analogous to our previous reports with native and recombinant TGF-β1 (2, 3), recombinant TGF-β3 had a biphasic stimulatory effect on DNA synthesis, enhanced collagen synthesis, and decreased alkaline phosphatase activity in osteoblast-enriched cultures after 23 h of treatment. TGF-β3 was more potent than TGF-β1, with an approximately three- to fivefold-lower concentration needed for similar half-maximal effects in all three assays.

Relative effects of TGF-β3 on DNA synthesis in osteoblast- and fibroblast-enriched bone cell populations. Earlier studies with isolated bone cells have revealed that TGF-β1 was a potent mitogen for cells within the osteoblast lineage, whereas minimal effects were seen in less differentiated fibroblast-enriched cultures from fetal rat bone (3). In the present studies, we observed that both TGF-β1 (data not shown) and TGF-β3 (Fig. 2) enhanced the rate of DNA synthesis to a greater extent in osteoblast-enriched cultures than in fibroblast-enriched bone cell cultures. In contrast, fetal bovine serum greatly increased the level of DNA synthesis in both cell populations (Fig. 2).

Receptor binding of TGF-β. Cross-linking studies have demonstrated that TGF-β1 associates with three distinct

FIG. 1. Effect of TGF-β3 (●) and TGF-β1 (○) on DNA synthesis (a), collagen synthesis (b), and alkaline phosphatase activity (c). Osteoblast-enriched cultures from fetal rat parietal bone were cultured to confluence and then serum deprived for 20 h prior to a 23-h treatment with either TGF-β3 or TGF-β1 at the concentrations shown. (a) DNA synthesis rates were measured by labeling cells with [^{3}H]thymidine for the last 2 h of culture; acid-insoluble material was assayed by scintillation counting. (b) Collagen synthesis was measured by labeling with [^{3}H]proline for the last 2 h of culture; acid-insoluble cell extracts were digested with nonspecific protease-free bacterial collagenase, and radioactivity was determined in the enzyme-released supernatants. (c) Alkaline phosphatase activity was measured in cell extracts by hydrolysis of *p*-nitrophenyl phosphate to p-nitrophenol (PNP). Data are the means ± SEM of four to six replicate cultures per condition.

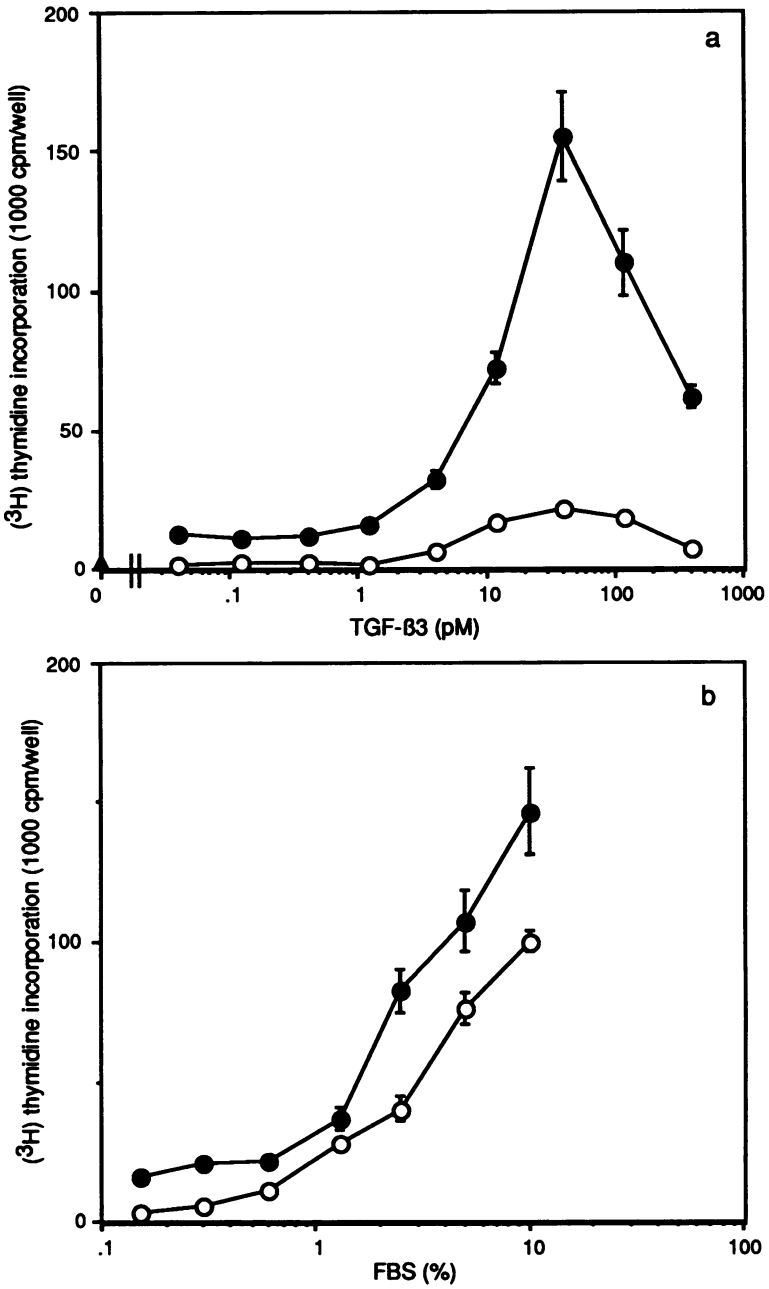


FIG. 2. Effect of TGF-β3 (a) and fetal bovine serum (b) on DNA synthesis of osteoblast-enriched (populations 3 to 5) (●) and fibroblast-enriched (population 1) (○) cells. Confluent cell cultures were serum deprived for 20 h prior to a 23-h treatment with TGF-β3 or fetal bovine serum (FBS) at the concentrations shown. DNA synthesis rates were measured by labeling cells with [^{3}H]thymidine for the last 2 h of culture; acid-insoluble material was assayed by scintillation counting. Data are the means ± SEM of four replicate cultures per condition.

classes of integral cell surface binding sites. We and others have previously reported TGF-β ligand-receptor complex types I, II, and III on fetal rat bone cells that correspond to bands of 65, 85, and >200 kDa when analyzed by polyacrylamide gel electrophoresis (2, 24). The type III receptor is most prominently labeled, followed by the type II and type I receptors, with approximate labeling ratios of 5:2:1, respectively. When [^{125}I]TGF-β1 and [^{125}I]TGF-β3 binding to osteoblast-enriched cultures was compared by these methods, we observed an identical labeling pattern by both ligands, and similar labeling ratios among the three binding sites (Fig. 3). Competition binding studies between [^{125}I]TGF-β1 (80 pM) and 40 to 2,560 pM unlabeled TGF-β1 and TGF-β3 demonstrated that TGF-β3 bound with a three- to fourfold-greater affinity (Fig. 4), and a significant displacement was observed for each receptor class (Fig. 5). The LIGAND program (19) was used to analyze the competition binding data and indicated an overall dissociation constant (K_d) of 200 pM for TGF-β1 and an overall inhibition constant (K_i) of 50 pM for TGF-β3.

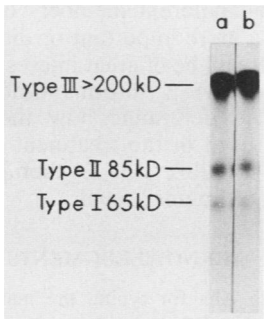


FIG. 3. Cell surface-associated binding of [^{125}I]TGF-β1 and [^{125}I] TGF-β3. A confluent monolayer of osteoblast-enriched cell culture was affinity labeled by incubation in the presence of [^{125}I]TGF-β1 (lane a) or [^{125}I]TGF-β3 (lane b), followed by treatment with disuccinimidyl suberate. Detergent-soluble cell extracts were displayed by gel electrophoresis and autoradiography. The positions of the three labeled TGF-β receptor types are indicated (in kilodaltons).

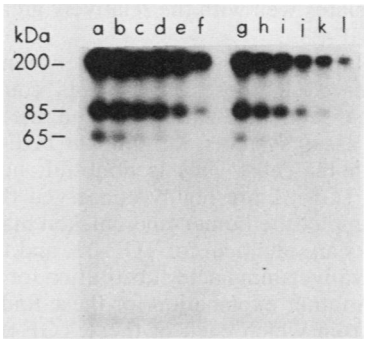


FIG. 5. Relative displacement at cell surface binding sites by TGF-β1 and TGF-β3 in osteoblast-enriched cultures form fetal rat bone. Confluent cell cultures were serum deprived for 20 h, rinsed, and incubated with 80 pM [^{125}I]TGF-β1 in the absence or presence of unlabeled TGF-β1 and TGF-β3 for 3 h at 4°C. The cell lysates were fractionated by electrophoresis on 7.5% polyacrylamide gels, and the bound ligand was visualized by autoradiography. Lane a, No addition; lanes b to f, unlabeled TGF-β1 at 80, 160, 320, 640, and 1,280 pM, respectively; lanes g to l, unlabeled TGF-β3 at 40, 80, 160, 320, 640, and 1,280 pM, respectively.

DISCUSSION

In the present studies we observed that TGF-β3 produces biochemical effects in fetal rat bone-derived cell cultures analogous to those of TGF-β1 (2–4). Like TGF-β1, TGF-β3 enhances DNA and collagen synthesis and decreases alkaline phosphatase activity in osteoblast-enriched cultures from fetal rat bone, but has minimal effects on DNA synthesis in fibroblastlike cells from the same tissue. As in our present and earlier studies with TGF-β1 (2–4), the mitogenic effect of TGF-β was biphasic, with significantly less activity at higher TGF-β concentrations. This may result from complex interactions between signals generated at different binding sites in these cultures. At lower TGF-β levels, the signal generated through the higher-affinity complex would predominate; at higher TGF-β concentrations, the moderating effect of a separate counteracting signal, produced by binding to the lower-affinity site, might then become evident. A more complete understanding of the intracellular signals induced by TGF-β occupancy at each binding site will be required to address this question directly, however.

Receptor-binding studies indicate that in osteoblast-enriched cell cultures, TGF-β3 associates with each of the three binding sites previously demonstrated with TGF-β1 and TGF-β2. The higher affinity of TGF-β3 compared with

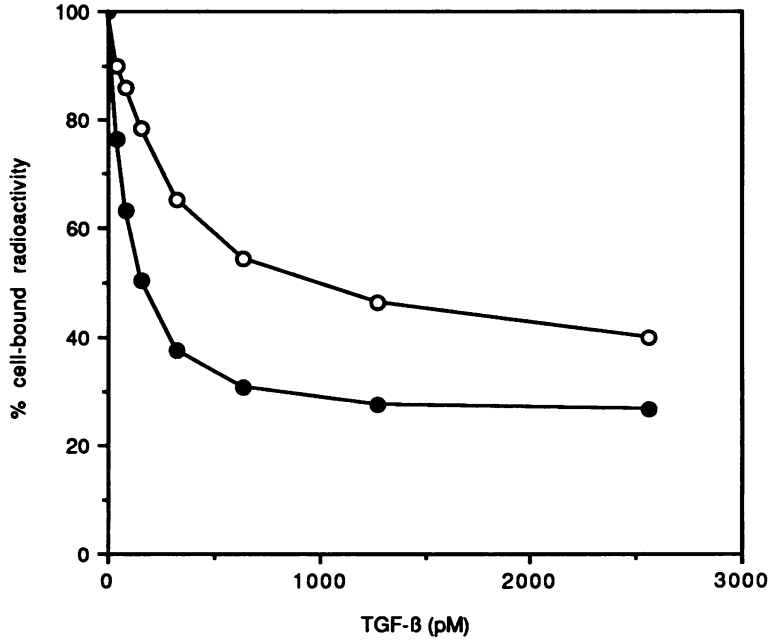


FIG. 4. Competitive binding between TGF-β1 and TGF-β3 in osteoblast-enriched cultures form fetal rat bone. Confluent cell cultures were serum deprived for 20 h, rinsed, and incubated with 80 pM [^{125}I]TGF-β1 in the presence of unlabeled TGF-β1 (○) or unlabeled TGF-β3 (●) for 3 h at 4°C. The cells were lysed, and the amount of bound radioligand was determined in a gamma counter. The maximum amount of [^{125}I] TGF-β1 bound in the absence of unlabeled competitor was 7,100 cpm, or 3.8% of its input concentration. The values shown are the average for duplicate culture wells and are representative of two separate experiments.

TGF-β1 correlates well with the relatively greater biological activity of TGF-β3.

Differences in activity and binding between TGF-β3 and TGF-β1 observed in these experiments could be due to subtle species sequence variations and the heterologous nature of the assay systems, i.e., human TGF-βs have been analyzed with rat cells. This is doubtful, however, since TGF-β3 and TGF-β1 are highly conserved through evolution. For example, the human and chicken mature polypeptide sequences are identical for TGF-β1, and there is only a single conservative amino acid substitution for TGF-β3 (8, 9, 12, 13, 28). Another explanation for these findings could be the sources from which each of these TGF-β preparations were acquired. TGF-β1, purified from human blood platelets, was obtained commercially, and recombinant TGF-β3 was purified to homogeneity from cell culture medium. We believe this second possibility is also unlikely due to the inherent stability of these proteins and because we have observed similar results with multiple preparations of the TGF-βs.

Recently, Graycar et al. (10) found that recombinant TGF-β3 has similar biological activities to TGF-β1 and -β2, but with different relative potencies depending on the cell type. Consistent with our data presented in this report and our observation on TGF-β3 growth inhibition of mink cells (K. K. Iwata, unpublished data), TGF-β3 has been found to be more potent than TGF-β1 in inhibiting DNA synthesis of mink cells and keratinocytes and more potent in stimulating DNA synthesis in AKR-2B cells (10). Receptor competition with [^{125}I]TGF-β1 showed that TGF-β1 competed more effectively than TGF-β3 in AKR-2B cells (10). In contrast, we observed that TGF-β3 competed more effectively than TGF-β1 in osteoblast cultures, an effect more consistent with its relatively greater biochemical potency. These differences could be due to experimental factors such as cell types, cell density, and method of radiolabeling.

Unlike our present results with TGF-β3, analogous studies comparing porcine blood platelet-derived TBF-β1 and TGF-β2 (R and D Systems, Inc., Minneapolis, Minn.) showed no consistently different effects on DNA synthesis or any correlation between mitogenic activity and ligand occupancy at type I TGF-β-binding sites in fetal rat osteoblast-enriched cultures (M. Centrella, unpublished results). This suggests that each of the TGF-βs differentially couples to binding sites and/or differs in the proportion of receptor ligand interaction resulting in postreceptor signals, but this possibility will require a better understanding of the nature of the TGF-β receptor that mediates increased DNA synthesis in these cells.

The precise role and mutual interactions of specific growth regulators in bone metabolism, such as the TGF-βs, fibroblast growth factors, platelet-derived growth factors, insulin-like growth factors, and the bone morphogenetic proteins, have yet to be determined. However, all TGF-β isoforms examined to date are potent mitogens for osteoblast-enriched cultures from fetal rat bone and enhance bone matrix protein synthesis (2–5, 22, 25, 27, 31; this study). Bone grows rapidly during development and early life. In addition, bone is continuously remodeled throughout the life span by cellular events that incorporate bone resorption and subsequent bone formation. Furthermore, in contrast to the healing process that occurs in most other connective tissues, bone repair involves the formation of new bone rather than scar tissue. TGF-β may stimulate precise steps in bone formation during each of the three physiological processes (development, remodeling, and fracture repair). It is possible, therefore, that different members of the TGF-β gene family may have a more important or direct role in each of these processes. It will be of great interest to understand the role of the various TGF-β isoforms in normal and aberrant bone formation, to determine how the different TGF-β isoforms may be used in the treatment of metabolic bone disease, and how to utilize TGF-βs therapeutically to accelerate the process of fracture repair.

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