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Biochemistry

Parathyroid hormone modulates transforming growth factor β activity and binding in osteoblast-enriched cell cultures from fetal rat parietal bone

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ABSTRACT **Transforming growth factor β (TGF-β) is produced by bone cells, is abundant in bone matrix, and regulates bone cell biochemical processes. In osteoblast-enriched fetal rat parietal bone cell cultures, low TGF-β doses increase DNA synthesis, whereas higher levels are less mitogenic, stimulate collagen production, and decrease alkaline phosphatase activity. Parathyroid hormone by itself has minimal effects on these processes, but it opposes the effects of TGF-β and alters TGF-β binding to its receptors in osteoblast-enriched cultures. Some functions ascribed to parathyroid hormone in bone may therefore result from alterations in TGF-β activity, suggesting that the local effects of TGF-β in bone are under systemic hormonal control.**

Although resorption is the most thoroughly studied effect of parathyroid hormone (PTH) on bone, the hormone also stimulates biochemical processes associated with bone formation (1–4). PTH does not directly affect bone-resorbing osteoclasts, and the osteoblast or its products appear to mediate all of the effects of PTH in bone (5–7). The mechanisms by which PTH regulates bone metabolism are incompletely known, but part of its action may involve cyclic AMP generation (1, 2, 8) and local growth factors (9–12). One local factor produced by bone cells that is abundant in bone matrix is transforming growth factor β (TGF-β) (11–13), and TGF-β regulates replication, collagen synthesis, and alkaline phosphatase in osteoblast-enriched cell cultures (13, 14). TGF-β content increases in bone culture medium after PTH treatment (15), and locally released TGF-β could account for the anabolic effects of the hormone on bone. The relationships among these factors in regulating bone formation are unknown. The present studies using osteoblast-enriched primary cell cultures address the combined effects of PTH and TGF-β on bone cell activity and the influence of PTH on the association of TGF-β with bone cell receptors.

MATERIALS AND METHODS

Growth Regulators. Recombinant human TGF-β (rhTGF-β; provided by H. Michael Shepard, Genentech, South San Francisco, CA) was stored at 20 μg/ml in 0.5 M acetic acid at 4°C. Parathyroid hormone (bovine PTH-(1–84), National Hormone and Pituitary Program, Baltimore; rat PTH-(1–34), Calbiochem) was solubilized at a concentration of 10^{-4} M in sterile water containing bovine serum albumin at 4 mg/ml and was stored at −70°C.

Cell Cultures. Parietal bones obtained from 22-day-old rat fetuses (Sprague–Dawley, Charles River Breeding Laboratories) were dissected free of sutures and were subjected to five 20-min collagenase digestions (14). Although some overlap is likely, the population released during the first collagenase treatment is enriched with fibroblasts, whereas the last three populations are enriched with cells expressing osteoblastic characteristics (2, 14). Cells were plated at 12,500 cells per cm^2 and were cultured to confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, ascorbic acid at 100 μg/ml, and 20 mM Hepes buffer (GIBCO); cells were serum-deprived for 20 hr, and test factors were added in serum-free medium for 23 hr as described (14, 16).

DNA Synthesis. To assess the mitogenic effects of the added agents, the rate of DNA synthesis was measured in 0.32-cm^2 cultures labeled with [^{3}H]thymidine (5 μCi/ml, 80 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) for the last 2 hr of culture. Cells were lysed in 0.1 M NaOH/0.1% sodium dodecyl sulfate and were acidified with 10% trichloroacetic acid, and acid-insoluble material was collected on glass fiber filters and quantitated by scintillation counting as described (14).

Collagen Synthesis. Collagen synthesis rates were measured in 2-cm^2 cultures labeled with [2,3-^{3}H]proline (25 μCi/ml, 250 mCi/mmol; New England Nuclear) for the last 2 hr of culture. Then 0.5% Triton X-100 (Sigma) cell lysates were extracted with 10% trichloroacetic acid/acetone, and the acid-insoluble material was digested with nonspecific protease-free bacterial collagenase. The amount of newly synthesized collagen was determined in the enzyme-released supernatants according to the method of Peterkovsky and Diegelmann (17).

Alkaline Phosphatase Activity. Alkaline phosphatase activity in 0.5% Triton X-100 cell extracts was determined by hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol, which was measured by spectroscopy at 410 nm as reported (14).

TGF-β Receptors. TGF-β receptors were examined by methods adapted from Massague and Like (18). Confluent cell cultures (9.6 cm^2) were serum deprived for 20 hr, rinsed, incubated for 1 hr at 37°C, rinsed, and incubated an additional hr at 4°C in serum-free medium containing 0.4% bovine serum albumin. ^{125}I-labeled TGF-β (^{125}I-TGF-β; 60 μCi/μg, 60 nCi/ml; Biomedical Technologies, Stoughton, MA), in the presence or absence of unlabeled TGF-β or PTH, was then added for 3 hr at 4°C. The cultures were rinsed with cold isotonic buffer (146 mM NaCl/11 mM dextrose/35 mM Tris·HCl, pH 7.4) and were crosslinked by a 15-min incubation at 4°C with 0.5 mM disuccinimidyl suberate (a stock solution of 50 mM was prepared in dimethyl sulfoxide). Cells were extracted with 1% Triton X-100 in isotonic buffer, and

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Abbreviations: PTH, parathyroid hormone; TGF-β, transforming growth factor β; rhTGF-β, recombinant human TGF-β; ^{125}I-TGF-β, ^{125}I-labeled TGF-β.

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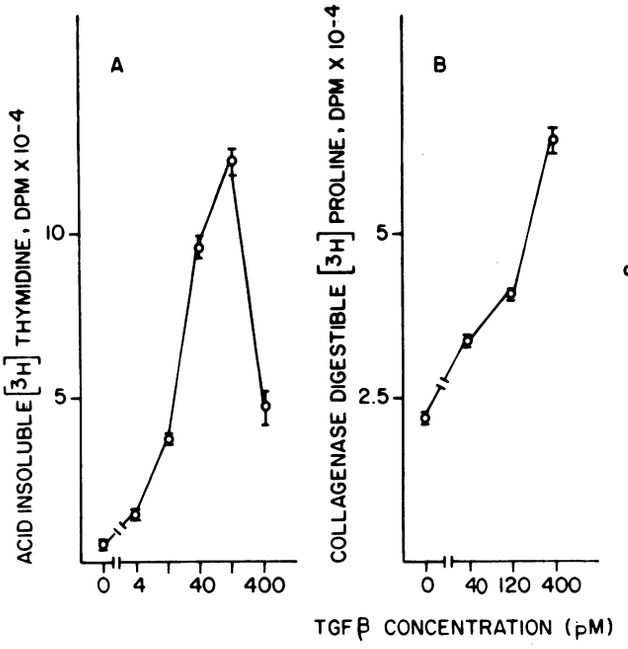

FIG. 1. Effect of rhTGF-β on DNA synthesis, collagen synthesis, and alkaline phosphatase activity in osteoblast-enriched cultures from fetal rat parietal bone. Cells were cultured to confluence and were serum deprived for 20 hr prior to a 23-hr treatment with TGF-β at the concentrations shown. (*A*) DNA synthesis rates were measured by labeling cells with [^{3}H]thymidine for the last 2 hr 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 hr of culture; acid-insoluble cell extracts were digested with nonspecific protease-free bacterial collagenase, and collagen synthesis 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 replicate cultures per condition.

nuclei were removed by centrifugation; supernatants were diluted with electrophoresis sample buffer containing 5% 2-mercaptoethanol and were electrophoresed through a 7.5% acrylamide gel according to Laemmli (19). ^{125}I-TGF-β-labeled bands were visualized by autoradiography. For mathematical analyses, some receptor studies were performed analogously in either 0.32- or 2-cm^2 culture wells, and the detergent-extracted cell lysates were directly quantitated in a γ spectrometer.

RESULTS

rhTGF-β had a biphasic stimulatory effect on DNA synthesis rates, stimulated collagen production, and inhibited alkaline phosphatase activity in osteoblast-enriched cultures obtained from fetal rat parietal bone (Fig. 1) with a potency analogous to human and porcine platelet TGF-β (14, 16). TGF-β had a dose-related effect on DNA synthesis rates at concentrations up to 40–120 pM, whereas higher concentrations were less stimulatory.

PTH-(1–84) had no effect on [^{3}H]thymidine incorporation at physiological concentrations of 0.02–0.2 nM (5) but inhibited basal DNA synthesis by 20–50% at 2–20 nM (Fig. 2). At submaximally mitogenic TGF-β concentrations (shown in Fig. 2 for 12 pM TGF-β), 2–20 nM PTH reduced TGF-β-stimulated DNA synthesis, and lower PTH levels had no effect. Analogous results were found at each submaximal dose of TGF-β examined up to 40 pM.

In contrast to the effects seen at submaximal TGF-β concentrations, the decreased level of mitogenesis seen with supramaximal TGF-β concentrations (shown for 400 pM TGF-β) was reversed by PTH. This reversing effect was near maximal at 0.2 nM PTH and declined at higher PTH doses (Fig. 2). It is important to note that low levels of PTH (in the range of 0.02–0.2 nM) were usually necessary to observe this reversing effect; in some experiments higher PTH levels produced a more obvious decline (for example, see Fig. 5) and, in the absence of lower test doses, would suggest no apparent effect by PTH.

By comparison, fibroblast-enriched cultures from fetal rat parietal bone are less sensitive to the mitogenic effects of TGF-β (14, 16); 20–200 nM PTH were required to modify TGF-β-mediated mitogenesis and produced less pronounced effects (data not shown).

PTH at 0.2 and 2 nM had no significant effects on basal collagen synthesis rates in the osteoblast-enriched cultures, whereas 20 nM PTH was slightly inhibitory. In contrast, 400

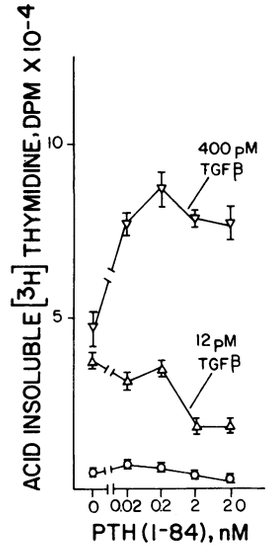

FIG. 2. Effect of PTH-(1–84) on DNA synthesis in TGF-β-treated osteoblast-enriched cultures from fetal rat parietal bone. Cell culturing, treatments, and [^{3}H]thymidine incorporation were as described in Fig. 1. Data are the means ± SEM of four replicate cultures per condition. ○, No TGF-β was added.

Table 1. Effect of PTH-(1–84) on collagen synthesis and alkaline phosphatase activity in TFG-β-treated osteoblast-enriched cell cultures

PTH-(1-84), nM	Collagen synthesis,* dpm × 10^{-3}		Alkaline phosphatase activity,† pmol·min^{-1}·μg^{-1}	
	− TGF-β	+ TFG-β	− TGF-β	+ TGF-β
0	21.9 ± 0.8	64.0 ± 2.4	693 ± 12	479 ± 17
0.2	22.2 ± 1.9	53.0 ± 4.8	712 ± 18	595 ± 19
2	19.1 ± 0.5	50.6 ± 2.9	700 ± 11	567 ± 26
20	17.8 ± 1.6	46.6 ± 1.8	689 ± 11	659 ± 11

rhTGF-β (400 pM) was used. Data are the means ± SEM of three replicate cultures per condition.

*[^{3}H]Proline incorporation into collagenase digestible protein was assayed as described in Fig. 1

†Alkaline phosphatase activity (pmol of *p*-nitrophenol released per min per μg of protein) was measured by hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol as described in Fig. 1.

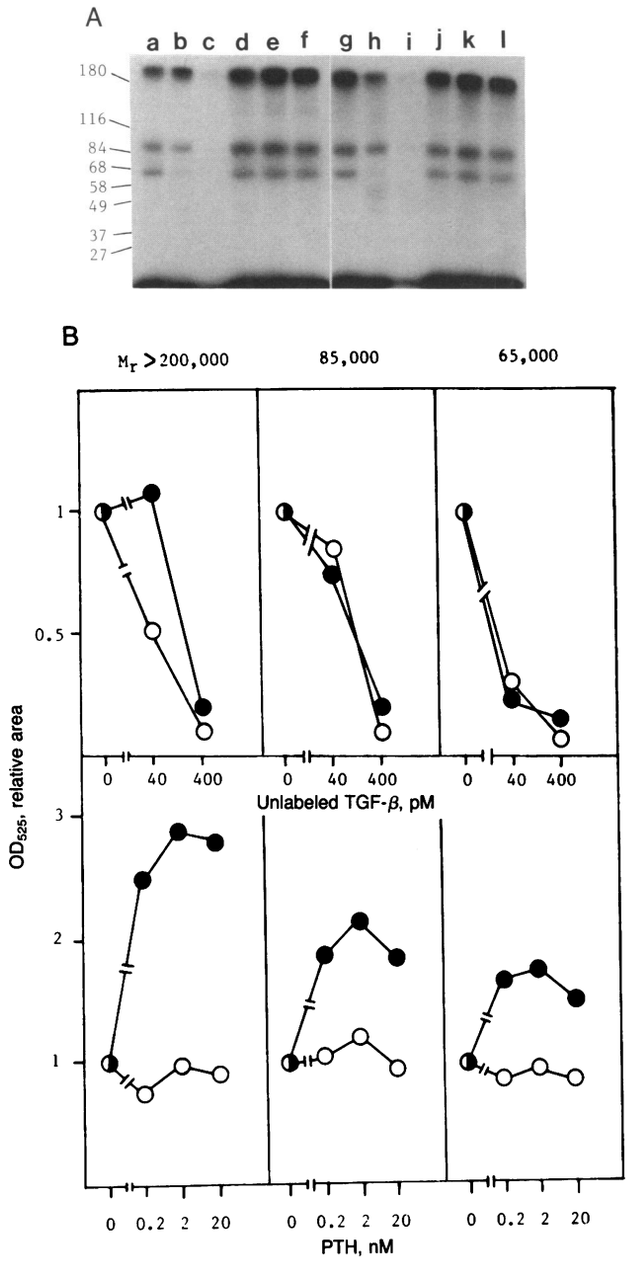

FIG. 3. Effect of PTH-(1–84) on TGF-β receptor binding in osteoblast- and fibroblast-enriched bone cell cultures. Confluent cell cultures were serum deprived for 20 hr, rinsed, and preincubated for 1 hr at 37°C and an additional 1 hr at 4°C prior to the addition of ^{125}I-TGF-β to a final concentration of 40 pM with or without unlabeled TGF-β or PTH-(1–84), all in serum-free medium containing 0.4% bovine serum albumin. After 3 hr at 4°C, cultures were rinsed and crosslinked with 0.5 mM disuccinimidyl suberate. Cells were extracted with 1% Triton X-100 in isotonic buffer, nuclei were removed by centrifugation, and supernatants were electrophoresed through a 7.5% acrylamide gel according to the method of Laemmli (19). (*A*) Autoradiograph of gel. Lanes: a and g, no addition; b and h, 40 pM rhTGF-β; c and i, 400 pM rhTGF-β; d and j, 200 pM PTH; e and k, 2 nM PTH; f and l, 20 nM PTH. Lanes: a–f, osteoblast-enriched cells; g–l, fibroblast-enriched cells. Molecular weight standards ($M_r \times 10^{-3}$) are indicated at left. (*B*) The amount of ^{125}I-TGF-β in each band was determined by densitometry in osteoblast-enriched (●) and fibroblast-enriched (○) cultures; data are shown as the density in the M_r >200,000, 85,000, and 65,000 receptor bands relative to binding in the absence of unlabeled TGF-β (*Upper*) or PTH (*Lower*).

pM TGF-β enhanced collagen synthesis 2.9-fold, and this increase was reduced 20% by 2 nM PTH. PTH also had no direct effect on alkaline phosphatase in these cells, but it

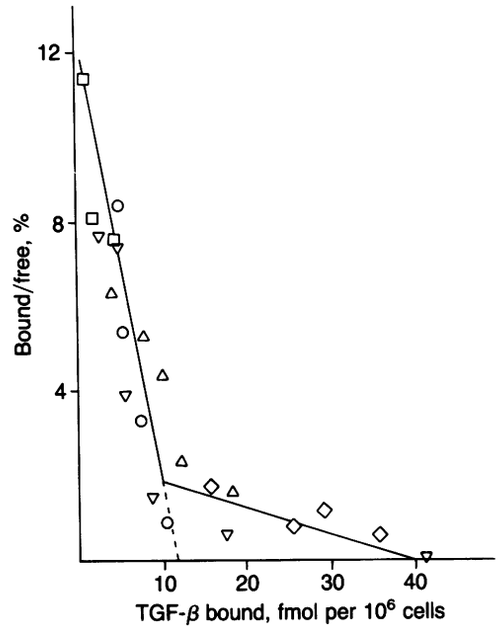

FIG. 4. Scatchard analysis of TGF-β receptors in osteoblast-enriched fetal rat parietal bone cell cultures. Cell cultures, treatments, and ^{125}I-TGF-β binding were as described in Fig. 3 with 40–400 pM ^{125}I-TGF-β and 40 pM–4 nM unlabeled TGF-β; nonspecifically bound TGF-β was determined with 100-fold or greater molar excess of unlabeled TGF-β and was routinely 15% of the total cpm bound in the absence of unlabeled ligand. Data are from five separate experiments with various amounts of labeled and unlabeled TGF-β to encompass the ranges of high- and low-affinity receptors. Each symbol represents an independent experiment.

opposed the decrease in enzyme activity that was induced by TGF-β (Table 1).

For receptor studies, cells were labeled with ^{125}I-TGF-β (human platelet TGF-β) in the presence or absence of unlabeled rhTGF-β or PTH. The level of receptor-bound ^{125}I-TGF-β was determined by chemical crosslinking, separation on polyacrylamide gels, autoradiography, and densitometry. As shown for other cells (18, 20), fibroblast- and osteoblast-enriched cultures from fetal rat parietal bone possessed three discrete TGF-β receptors of M_r values >200,000, 85,000, and 65,000. The most prominently labeled polypeptide in both cultures was of M_r >200,000. Unlabeled rhTGF-β competed with ^{125}I-TGF-β for all receptors and displaced binding of ^{125}I-TGF-β at the M_r 65,000 receptor the most effectively in both cultures; displacement required more unlabeled TGF-β at the M_r 85,000 receptor, but it was also equivalent in both cultures (Fig. 3). Therefore, the fibroblast- and osteoblast-enriched cultures each possessed similar high-affinity M_r 65,000 receptors and lower affinity M_r 85,000 receptors. In

Table 2. Apparent abundance and dissociation constants (K_d) for high- and low-affinity TGF-β receptors in fibroblast-enriched and osteoblast-enriched parietal bone cell cultures

	Receptor number $\times 10^{-4}$		K_d, pM	
Receptor	Fib	Ost	Fib	Ost
High affinity	0.5–0.9	0.5–1.0	50–100	50–100
Low affinity	1.7–2.0	1.8–2.7	370–400	1100–1200

Apparent receptor number and K_d were determined by Scatchard analysis with 40–400 pM ^{125}I-TGF-β and 40 pM–4 nM unlabeled TGF-β with confluent cell cultures, as depicted in Fig. 4. Specifically bound TGF-β was determined after subtracting nonspecific background binding (15% of cpm bound in absence of unlabeled ligand). Fib, Fibroblast-enriched cultures; Ost, osteoblast-enriched cultures. Data were obtained from five independent experiments.

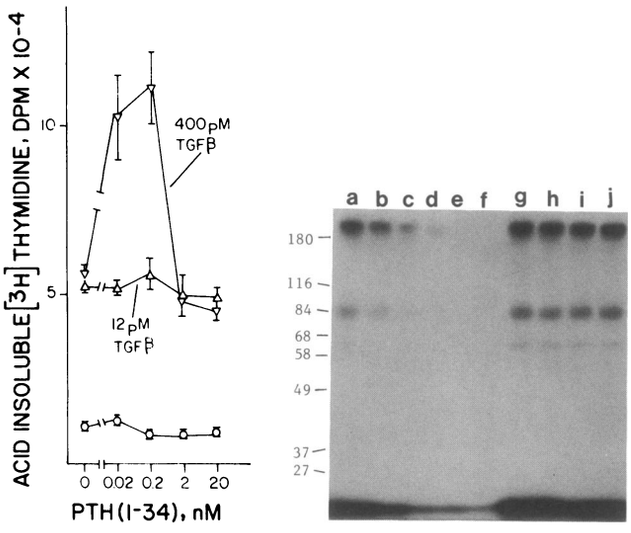

FIG. 5. Effect of PTH-(1–34) on TGF-β receptor binding and mitogenesis in osteoblast-enriched fetal rat parietal bone cell cultures. (*Left*) DNA synthesis was assayed as described in Fig. 1. ○, No TGF-β was added. (*Right*) TGF-β binding was as described in Fig. 3 with 40 pM ^{125}I-TGF-β. Lanes: a, no addition; b, 40 pM TGF-β; c, 120 pM TGF-β; d, 400 pM TGF-β; e, 1.2 nM TGF-β; f, 4 nM TGF-β; g, 20 pM PTH-(1–34); h, 200 pM PTH-(1–34); i, 2 nM PTH-(1–34); j, 20 nM PTH-(1–34). Molecular weight standards ($M_r \times 10^{-3}$) are indicated at left.

contrast, binding at the M_r >200,000 receptor relative to the M_r 65,000 receptor differed in both cultures. By comparison to the fibroblast-enriched cultures, displacement of labeled TGF-β at the M_r >200,000 receptor in the osteoblast-enriched cultures required more unlabeled ligand, which suggested reduced ligand affinity at this site (Fig. 3). Scatchard analysis was curvilinear and indicated a similar total number of high-affinity TGF-β receptors in both cell populations. However, low-affinity receptors in the osteoblast-enriched cultures had ≈3-fold lower ligand affinity relative to the fibroblast-enriched cells (Fig. 4 and Table 2).

PTH enhanced TGF-β binding in the osteoblast-enriched cultures, but it had no notable effect on TGF-β binding in the fibroblast-enriched cultures. Densitometry showed that PTH induced a dose-related increase in TGF-β binding at each receptor band in the osteoblast-enriched cultures, and this effect was more evident in low-affinity receptors (Fig. 3). Similar results were found with the 1-34 amino-terminal fragment of rat PTH (Calbiochem); levels as low as 20 pM rat PTH-(1–34) increased TGF-β binding and, like the 84-amino acid polypeptide, augmented the reduced mitogenic activity seen at supramaximal TGF-β concentrations (Fig. 5).

Table 3. Apparent abundance and K_d for TGF-β receptors in osteoblast-enriched parietal bone cell cultures treated with PTH

Receptor	Receptor number × 10^{-4}		K_d, pM	
	− PTH	+ PTH	− PTH	+ PTH
High affinity	0.5	0.8	100	210
Low affinity	2.2	3.7	1190	1670

Apparent receptor number and K_d were determined by Scatchard analysis by using 40–2000 pM ^{125}I-TGF-β with confluent 0.32-cm^2 osteoblast-enriched cultures, as depicted in Fig. 6. Specifically bound TGF-β was determined after subtracting 15% nonspecific background binding. Rat PTH-(1–34) (20 pM) was used.

The effect of PTH on TGF-β binding was also quantitated by total ^{125}I-TGF-β binding and by Scatchard analysis obtained by using progressively greater amounts of ^{125}I-TGF-β; 20 pM rat PTH-(1–34) increased binding at high- and low-affinity TGF-β receptors about 1.5-fold (Fig. 6 and Table 3).

To examine if longer PTH treatment could produce a stable change in the TGF-β receptor profile, each bone cell population was pretreated for 20 hr at 37°C with 0.2–20 nM PTH, washed, and then incubated with ^{125}I-TGF-β in the absence of PTH; no changes in TGF-β binding were detected by autoradiography (data not shown).

DISCUSSION

TGF-β has been isolated from bone culture medium and bone matrix extracts (11, 12), and its concentration is increased by agents, such as PTH, that stimulate bone resorption (15). Earlier results have shown that TGF-β produces concentration-dependent effects on replication (DNA synthesis and cell number increase) and expression of differentiated function (collagen synthesis and alkaline phosphatase activity) in osteoblast-enriched cell cultures (13, 14, 16). The present studies further indicate that PTH tends to oppose the effects of TGF-β on each of these processes. At lower concentrations of TGF-β, its mitogenic effect is reduced by relatively high PTH concentrations. At higher, less stimulatory TGF-β concentrations, low amounts of PTH increase mitogenesis; in this situation, both the rate of collagen synthesis and alkaline phosphatase activity revert toward the levels detected at the lower, but more mitogenic, TGF-β concentrations. Control of TGF-β activity by PTH, therefore, may be a mechanism used by osteoblasts to generate a balance between the effects of these factors when both are accumulating in bone tissue during remodeling.

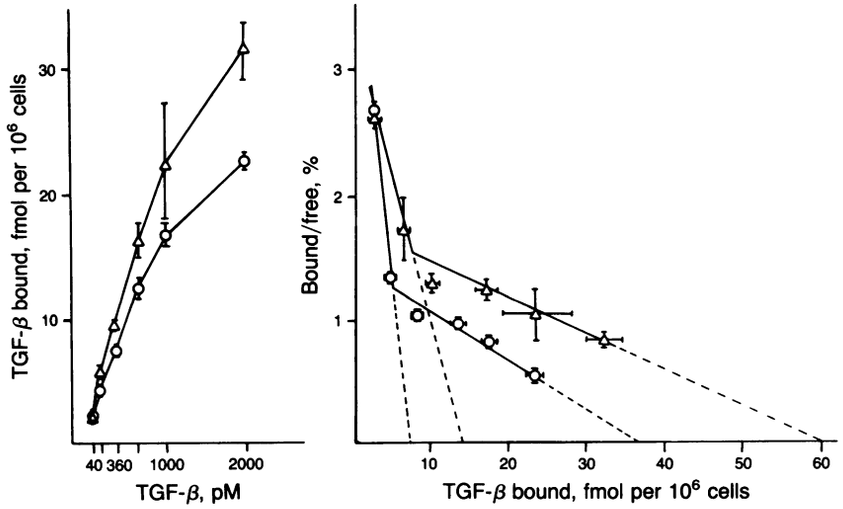

FIG. 6. Effect of PTH-(1–34) on Scatchard analysis of TGF-β binding in osteoblast-enriched fetal rat parietal bone cell cultures. Cell cultures, treatments, and ^{125}I-TGF-β binding were determined in 0.32-cm^2 cultures as described in Fig. 3 with 40–2000 pM ^{125}I-TGF-β; specific TGF-β binding was determined after subtracting 15% nonspecific background binding. Data are shown as the means ± SEM of triplicate determinations in the absence (○) or presence (△) of 20 pM rat PTH-(1–34).

It is unlikely that the effects of PTH at submaximal and supramaximal TGF-β concentrations result from the same PTH-generated signals since they occur at very different PTH concentrations. PTH effects conceivably are mediated through more than one postreceptor event. Other studies have suggested that some PTH effects may occur as a result of an increase in cyclic AMP accumulation (8), whereas additional effects may be determined by changes in the intracellular calcium concentration (21). These possibilities could account for the dissimilar effects of PTH at submaximal and supramaximal TGF-β concentrations. Although the mechanism(s) that regulates each of these results is not yet certain, PTH does not appear to alter the level of TGF-β mRNA in these cell cultures during the time frame of these studies (T.L.M., unpublished results).

This report describes studies with human recombinant platelet TGF-β, but identical results have also been obtained with native human platelet TGF-β; in addition, the effects of TGF-β on [^{3}H]thymidine incorporation into DNA, in the presence and absence of PTH, are paralleled by changes in cell number (M.C., unpublished results).

TGF-β is significantly concentrated in bone tissue (12, 22), and the local amount of TGF-β available to bone cells is likely to be higher than that present in serum. Also, the level of TGF-β in culture medium conditioned by fetal rat calvariae (22) is about 10-fold greater than that in culture medium conditioned by retrovirus-transformed rat cells (23). Therefore, the effects seen in the present studies at higher TGF-β levels may be physiologically relevant in bone systems. These effects are detected well below the range of the calculated dissociation constants for low-affinity TGF-β receptors in osteoblast-enriched cell cultures (at ≈20% receptor occupancy), and near physiological PTH concentrations appear to affect binding predominantly at these sites.

The pattern of TGF-β receptor labeling by these bone cells is similar to that for other fibroblast and mesenchymal tissue-derived cell cultures, where the M_r >200,000 receptor predominates. The abundance and dissociation constants for both high- and low-affinity TGF-β receptors in the fibroblast-enriched cultures from parietal bone are similar to those reported for nonskeletal fibroblasts (18, 20). The M_r >200,000 receptor in the osteoblast-enriched cell cultures appears to bind TGF-β with lower affinity relative to the fibroblast-enriched cultures and probably accounts for the higher dissociation constant for low-affinity receptors calculated for these cells. Since PTH increases TGF-β binding during a 3-hr incubation at 4°C, conditions under which virtually no protein biosynthesis occurs, this likely reflects an alteration in ligand binding independent of new receptor synthesis. However, Scatchard analysis indicates an increase in the abundance of both high- and low-affinity TGF-β receptors in the presence of PTH, with a higher concentration of TGF-β needed for receptor saturation. This apparent increase in receptor number may be due in part to the complexity inherent in mathematical analysis of multiple receptors with various affinities. Alternately, preexisting TGF-β receptors may be unmasked by some presently unknown mechanism and thereby accommodate greater amounts of TGF-β.

Receptor binding is measured soon after exposure to TGF-β, and biochemical effects are assayed many hours later; consequently, the increase in TGF-β binding associated with PTH treatment may not directly or totally be responsible for all of the biochemical effects observed. Changes in cellular activity may depend on the net level of primary signals generated in the presence of both factors, as well as on secondary effects specifically and independently generated by each agent. Unlike some other growth promoters, TGF-β does not appear to induce receptor autophosphorylation (24). However, PTH stimulates cyclic AMP accumulation, an effect that is much greater in the osteoblast- than the fibroblast-enriched cultures, and other agents that increase intracellular cyclic AMP appear to regulate the biochemical effects of TGF-β in these cells in a similar way (M.C., unpublished results). Therefore, some effects induced by this combination of agents may result from changes in cyclic AMP-dependent phosphokinase or phosphatase activities that could control TGF-β–receptor interactions or some subsequent biochemical events, but this has not yet been investigated.

The relationship of PTH and TGF-β to other bone cell activities is unknown, and these results do not explain all the effects of PTH in bone metabolism; nevertheless, they support an indirect anabolic role, mediated by TGF-β, for this hormone in bone remodeling.

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