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Modulation of type β transforming growth factor activity in bone cultures by osteotropic hormones

JOHANNES PFEILSCHIFTER AND GREGORY R. MUNDY

Department of Medicine/Endocrinology, University of Texas Health Science Center, San Antonio, TX 78284

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ABSTRACT **Type β transforming growth factor (TGF-β) activity was found in conditioned medium harvested from fetal rat and neonatal mouse calvariae by using the anchorage-independent growth of normal rat kidney fibroblasts as an indicator system. Calvariae incubated with parathyroid hormone, 1,25-dihydroxyvitamin D_3, and interleukin 1, all factors that stimulate bone resorption, showed a concentration-dependent increase in TGF-β activity in the culture medium. Increases in TGF-β activity persisted undiminished for 48 hr after removal of these factors. The increases in TGF-β activity from the resorbing bone cultures were relatively greater than the increases in bone resorption. Calcitonin inhibition of bone resorption correlated with a decrease in TGF-β activity. Thus, agents that modulate bone resorption also affect TGF-β activity in the bone culture medium. Changes in local concentrations of TGF-β activity by osteotropic hormones may be important in the regulation of normal bone remodeling.**

Transforming growth factors (TGFs) are operationally defined by their ability to induce anchorage-independent growth of cells in soft agar (1–3). By using normal rat kidney (NRK) fibroblasts as indicator cells, two distinct classes of TGFs have been identified. Type α TGF (TGF-α) is a structural analog of epidermal growth factor (EGF) and binds to the EGF receptor (4, 5). TGF-α induces the formation of small colonies of NRK cells in soft agar. Type β TGF (TGF-β) does not bind to the EGF receptor and differs from TGF-α in molecular composition. TGF-β alone does not induce anchorage-independent growth of NRK cells but potentiates the effects of TGF-α or EGF on colony formation (6, 7). Unlike TGF-α, which has been demonstrated so far only in neoplastic and embryonic tissues (8, 9), TGF-β has also been detected in many normal nonneoplastic adult tissues (6, 10, 11).

Recently, it has been realized that bone is a rich source of TGF-β. Conditioned medium from fetal rat calvaria cultures contains TGF-β activity (12), and TGF-β is abundant in demineralized bone matrix (13). Local growth regulation in bone is characterized by a localized remodeling, in which periods of bone resorption are followed by subsequent new bone formation that serves to repair the bone defect caused by resorption (14). It is likely that the coupling of bone formation to bone resorption is mediated by local factors in the bone microenvironment that act as regulatory growth factors for osteoblasts, and it has recently been suggested that TGF-β affects osteoblast function (15–17). We therefore tested whether TGF-β activity in bone cultures could be influenced by the osteotropic factors parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], interleukin 1 (IL-1), and calcitonin (CT).

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MATERIALS AND METHODS

Organ Culture of Bone. Calvariae (frontal and parietal bones) from 21-day fetal rats or 4-day-old mice were dissected aseptically and cultured on steel grids at the interface between 1 ml of medium and air in 12-well plastic culture dishes (Corning, Medfield, MA). The bones were maintained in BGJ medium (Irvine Scientific, Santa Ana, CA) containing either 5% heat-inactivated fetal bovine serum (GIBCO) or 1 mg of bovine serum albumin (Sigma) per ml. A 24-hr preincubation in control medium preceded all experiments. The calvariae were then incubated in fresh medium containing bone resorbing factors or corresponding control medium. After 48 hr of incubation with the calvariae, this medium was collected, and ^{45}Ca release and TGF-β activity in the medium were determined as described below.

In a second set of experiments the calvariae were incubated for 48 hr in medium containing bone resorbing factors or corresponding control medium, then rinsed extensively in phosphate-buffered saline, and cultured for another 4–24 hr in control medium to free them from the presence of the bone resorbing agents. At this point, the calvariae were incubated again in fresh control medium containing no bone resorbing factors and this medium was collected after 48 hr of incubation with the calvariae and analyzed as described below.

In some experiments, conditioned medium from untreated mouse calvariae was collected after 48 hr and pooled, and bone resorbing factors were added in the same concentrations used in the bone cultures described above. The conditioned media and the freshly added hormones at each concentration ($n = 8$ for each concentration) were then tested for their effects in the soft agar transformation assay.

Measurement of Bone Resorption. Calvariae of neonatal mice were prelabeled with ^{45}Ca by injecting the mothers 3–4 days before delivery. ^{45}Ca released into the medium and ^{45}Ca remaining in the calvariae were measured by scintillation counting and bone resorption was determined as the percentage of ^{45}Ca released into the medium during the culture period.

Soft Agar Transformation Assay. Anchorage-independent growth was determined by using a modification of the methods developed by Todaro, Massagué and others (1, 9, 18). One milliliter of Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 10% fetal bovine serum, 1% 1 M Hepes (pH 7.4) (Sigma), 1% nonessential amino acids (GIBCO), and 0.8% agarose (Sea Plaque, Marine Colloids Division, FMC, Rockland, ME) was pipetted into 35-mm Petri dishes (Corning). This underlay was allowed to solidify while a single cell suspension of NRK fibroblasts (clone 49F) (obtained from the American Type Culture Collection) was prepared. Cells were suspended in the above mentioned

Abbreviations: TGF, transforming growth factor; TGF-β, type β TGF; EGF, epidermal growth factor; BDGF, bone-derived growth factor; SGF, skeletal growth factor; PTH, parathyroid hormone; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; IL-1, interleukin 1; CT, calcitonin; NRK, normal rat kidney; U, unit(s).

Table 1. Conditioned medium from fetal rat or neonatal mouse calvariae promotes anchorage-independent growth of NRK 49F cells incubated with EGF

Colonies ≥ 100 μm	Medium alone	Fetal rat calvariae		Neonatal mouse calvariae	
		Without PTH	With PTH	Without PTH	With PTH
Without EGF	0	0	0	0	0
With EGF	6 ± 1	60 ± 7	96 ± 5*	42 ± 3	170 ± 19†

Fetal rat or neonatal mouse calvariae were incubated with or without 200 ng of PTH per ml. Forty-eight-hour conditioned culture medium was tested on its capacity to promote anchorage-independent growth of NRK cells with or without 2 ng of EGF per ml. The effect of medium alone (not incubated with calvariae) is shown in the second column. Each culture medium was assayed in duplicate. Colony numbers are mean ± SEM from five independent samples.
*Significantly different from the "Without PTH" group: $P < 0.005$.
†Significantly different from the "Without PTH" group: $P < 0.001$.

DMEM mixture to give a final concentration of 1×10^4 cells per ml and 0.5 ml of the suspension was added to the underlay. In most experiments EGF was added to a final concentration of 2 ng/ml. The overlay was allowed to solidify and 0.25-ml aliquots of conditioned medium from the bone cultures were added. Cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 7–10 days, the total number of colonies ≥ 100 μm in diameter was counted in each plate by using a light microscope with an eyepiece micrometer. Samples were assayed in duplicate. TGF-β activity was assessed as the number of colonies ≥ 100 μm in diameter that were induced with conditioned medium in the presence of 2 ng of EGF per ml.

A standard curve with purified platelet TGF-β was performed with each soft agar assay. TGF-β was tested in concentrations from 40 pg/ml to 600 pg/ml, and within that range colony numbers increased in a linear manner with increasing TGF-β concentrations. For measuring TGF-β activity in conditioned medium, aliquots of the medium were taken that induced colony numbers within the linear range determined by the TGF-β standard.

Statistical Analysis. Statistical differences were determined by using Student's *t* test for unpaired samples.

Hormones and Factors. Human PTH-(1–34) was purchased from Bachem (Torrance, CA). Recombinant human IL-1 [specific activity, 6×10^6 units (U)/mg] was a generous gift of P. Lomedico (Hoffman–LaRoche). 1,25-$(OH)_2D_3$ was provided by M. Uskokovic (Hoffman–LaRoche). Salmon CT was from Armour (Kankakee, IL). EGF was obtained from Collaborative Research (Lexington, MA). Purified TGF-β from human platelets was kindly provided by M. Sporn and A. Roberts (National Cancer Institute, Bethesda, MD).

RESULTS

TGF-β Activity in Medium from Rat and Mouse Calvariae. Conditioned medium from fetal rat calvariae and neonatal mouse calvariae stimulated colony formation of NRK cells in the presence of 2 ng of EGF per ml but did not induce colony formation without EGF, thus indicating the presence of TGF-β activity (Table 1). EGF alone induced numerous small colonies but only a few colonies larger than 100 μm in diameter. Conditioned medium harvested from 48-hr bone cultures stimulated colony formation to the same degree as 0.3–0.4 ng of platelet-purified TGF-β per ml.

TGF-β Activity and Bone Resorbing Agents. When calvariae were incubated with 200 ng of PTH per ml, TGF-β activity increased in the culture medium (Table 1). Increases in TGF-β activity with PTH were larger in medium from neonatal mouse calvariae than in medium from fetal rat calvariae. Previous experiments had shown that mouse calvariae also resorb far more in response to PTH than rat calvariae (unpublished observations). To study the relationship between TGF-β activity and bone resorption in response to bone resorbing agents, we therefore performed the following experiments in mouse calvariae.

PTH, 1,25-$(OH)_2D_3$, and IL-1 are all potent stimulators of bone resorption *in vitro* (19–21). Incubation of mouse calvariae with these factors induced concentration-dependent increases in ^{45}Ca release that persisted for 48 hr after removal of these factors (Figs. 1–3). Increases in bone resorption were accompanied in each instance by increases of TGF-β activity in the culture medium (Figs. 1–3). There was, however, no perfect correlation between both parameters. Increases in TGF-β activity with high concentrations of PTH (200 ng/ml) and IL-1 (100 U/ml) exceeded increases in ^{45}Ca release by up to 4-fold. TGF-β activity during the first 48-hr incubation period still increased at concentrations of IL-1 (100 U/ml) and PTH (200 ng/ml) at which there was no further increase in bone resorption.

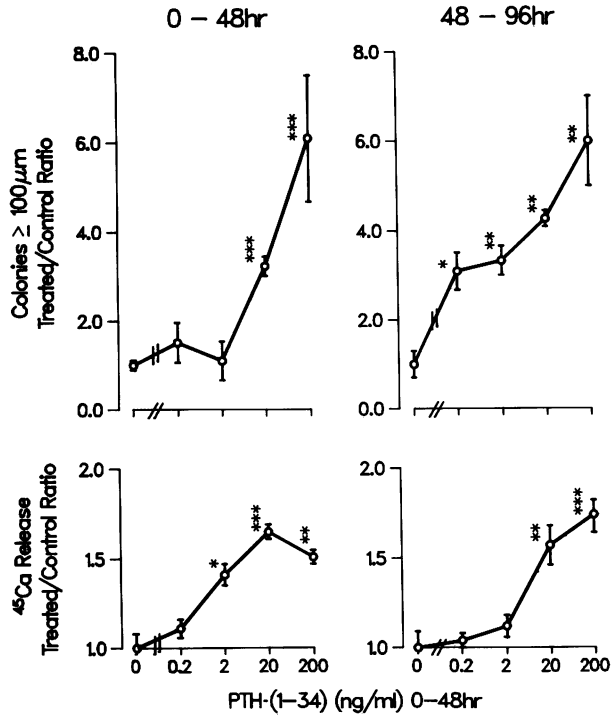

FIG. 1. Dose–response curves for the effects of PTH on bone resorption in neonatal mouse calvariae (*Lower*) and on TGF-β activity in the culture medium from these calvariae (*Upper*). The TGF-β assay was based on stimulation of colony growth of NRK cells incubated with EGF. The total number of colonies ≥ 100 μm was corrected for colony growth with EGF alone. Data are mean ± SEM from four bone cultures and are expressed as treated/control ratios. Either medium was analyzed 48 hr after the addition of PTH (*Left*) or calvariae were transferred to PTH-free medium after a 48-hr preincubation period with PTH and the medium was analyzed 48 hr later (*Right*). Significantly different from control (0): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

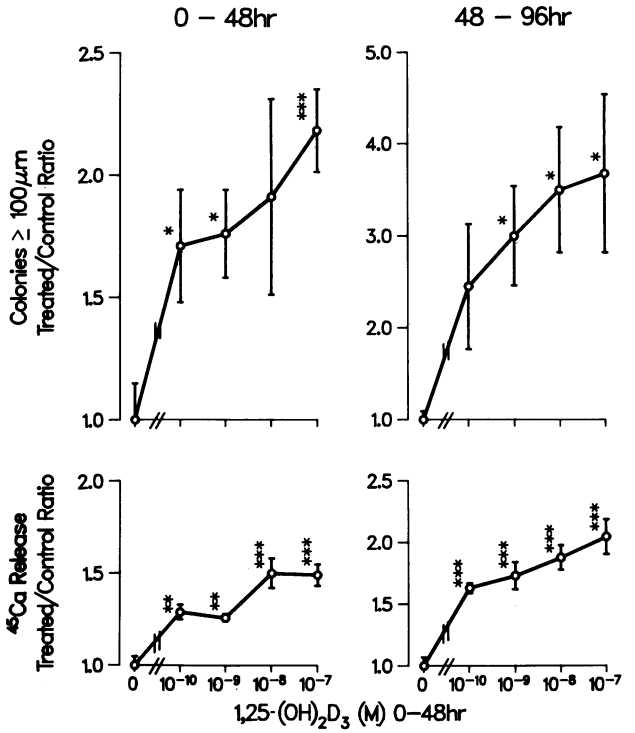

FIG. 2. Dose–response curves for the effects of 1,25-$(OH)_2D_3$ on bone resorption in neonatal mouse calvariae (*Lower*) and on TGF-β activity in the culture medium from these calvariae (*Upper*). The total number of colonies ≥ 100 μm was corrected for colony growth with EGF alone. Data are mean ± SEM from four bone cultures and are expressed as treated/control ratios. Either medium was analyzed 48 hr after the addition of 1,25-$(OH)_2D_3$ (*Left*) or calvariae were transferred to 1,25-$(OH)_2D_3$-free medium after a 48-hr preincubation period with 1,25-$(OH)_2D_3$ and the medium was analyzed 48 hr later (*Right*). Significantly different from control (0): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

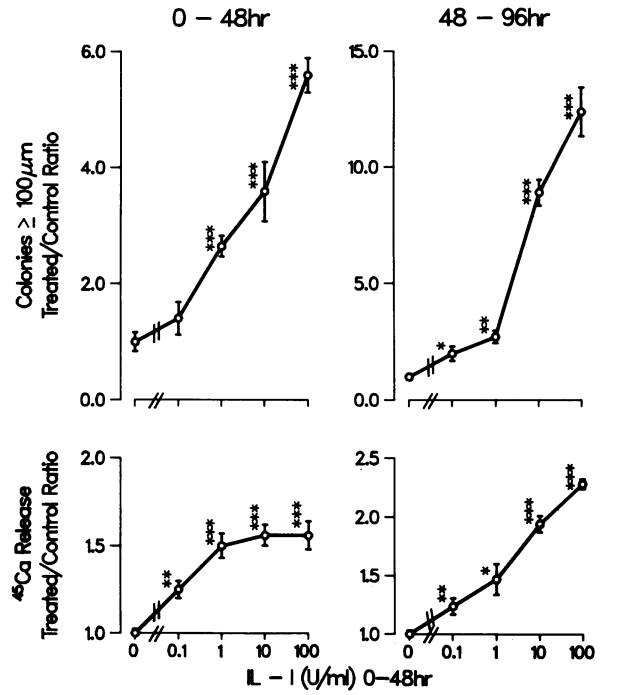

FIG. 3. Dose–response curves for the effects of IL-1 on bone resorption in neonatal mouse calvariae (*Lower*) and on TGF-β activity in the culture medium from these calvariae (*Upper*). The total number of colonies ≥ 100 μm was corrected for colony growth with EGF alone. Data are mean ± SEM from four bone cultures and are expressed as treated/control ratios. Either medium was analyzed 48 hr after the addition of IL-1 (*Left*) or calvariae were transferred to IL-1-free medium after a 48-hr preincubation period with IL-1 and the medium was analyzed 48 hr later (*Right*). Significantly different from control (0): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TGF-β Activity and CT. CT is a potent inhibitor of osteoclastic bone resorption (22), but there is no evidence that it inhibits effects of bone resorbing agents on cells other than osteoclasts. CT nearly completely inhibited PTH-induced increases in bone resorption (Fig. 4). It also partially inhibited the PTH-induced increases in TGF-β activity in the calvarial culture medium. However, there was still a >2-fold increase in TGF-β activity compared with control conditioned medium. When added to otherwise untreated calvariae, CT decreased ^{45}Ca release by 40% and decreased TGF-β activity in these cultures by 50%.

Effects of Osteotropic Hormones on TGF-β Measurement. PTH, 1,25-$(OH)_2D_3$, and CT at all concentrations used in the above experiments had no direct effect on colony growth of NRK cells in soft agar (Table 2). IL-1 in concentrations of 100 U/ml stimulated colony numbers 2-fold when added directly to the soft agar assay together with conditioned medium from untreated calvariae. However, these combined effects of IL-1 and TGF-β from the bone conditioned medium were only observed at 100 U of IL-1 per ml and cannot account for the increases in colony numbers observed with IL-1-free conditioned medium from calvariae pretreated with IL-1.

DISCUSSION

The data reported in this study show that TGF-β activity appears in conditioned medium from rat and mouse calvariae and that in mouse calvariae bone resorbing factors increase TGF-β activity in medium harvested from resorbing bones. We also found that TGF-β activity in the bone culture medium was less when bone resorption was inhibited with CT.

Our assay, a widely used indicator system for TGF-β activity, is based on TGF-β stimulation of anchorage-independent growth of fibroblasts. We used the kidney fibroblast cell line NRK 49F in which TGF-β promotes colony growth only with addition of EGF (7). Using 2 ng of EGF per ml and 10% fetal bovine serum, we were able to induce colony growth with <20 pg of purified platelet TGF-β per ml and colony numbers increased in a linear manner with TGF-β concentrations from 40 pg/ml to 600 pg/ml. The soft agar

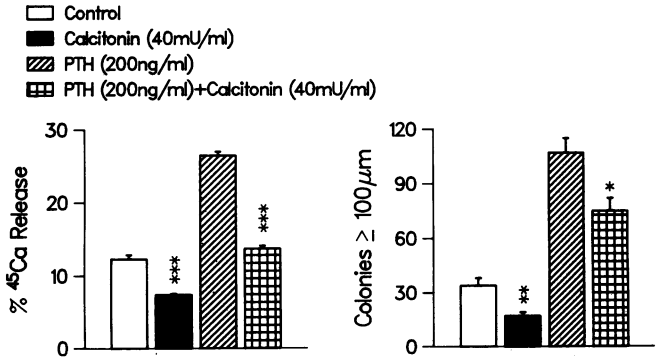

FIG. 4. Effects of CT (40 mU/ml) on bone resorption (*Left*) and TGF-β activity in the culture medium (*Right*) of calvariae incubated with or without PTH (200 ng/ml). The total number of colonies ≥ 100 μm was corrected for colony growth with EGF alone. Data are mean ± SEM from seven cultures. Medium was analyzed 48 hr after addition of the hormones. Significantly different from CT untreated reference group: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.01$.

Table 2. Influence of osteotropic agents on anchorage-independent growth of NRK 49F cells incubated in conditioned medium from mouse calvariae and 2 ng of EGF per ml

PTH, ng/ml	Colonies ≥ 100 μm	$1,25\text{-}(OH)_2D_3$, M	Colonies ≥ 100 μm	IL-1, U/ml	Colonies ≥ 100 μm	CT, mU/ml	Colonies ≥ 100 μm
0	71 ± 9	0	93 ± 8	0	91 ± 19	0	82 ± 12
0.2	75 ± 9	10^{-10}	105 ± 4	0.1	114 ± 24	40	71 ± 6
2	64 ± 7	10^{-9}	95 ± 3	1	92 ± 24		
20	56 ± 8	10^{-8}	99 ± 2	10	96 ± 20		
200	59 ± 5	10^{-7}	84 ± 9	100	235 ± 30*		

Forty-eight-hour conditioned medium from untreated mouse calvariae was collected, and PTH, $1,25\text{-}(OH)_2D_3$, IL-1, or CT was then added to the indicated concentrations. Supplemented medium (0.25 ml) was added to NRK cells that were cultured in soft agar in the presence of 2 ng of EGF per ml. Data represent eight replicates ± SEM. The total number of colonies ≥ 100 μm was corrected for colony growth with EGF alone.

*Significantly different from control (0): $P < 0.001$.

transformation assay is therefore capable of detecting very low concentrations of TGF-β activity as well as small differences in TGF-β activity.

The growth of colonies in soft agar may reflect complex interactions between several different growth factors. The effects of most growth factors on the growth of NRK cells in soft agar depend to a large extent on the assay conditions. TGF-β is the only growth factor known to cause markedly increased responses (10-fold or more) on the formation of NRK colonies in 10% fetal bovine serum. Colony growth is influenced by factors such as platelet-derived growth factor (PDGF), insulin-like growth factor II (IGF-II), and fibroblast growth factor (FGF) (23–26) found in fetal bovine serum. Depletion of these factors from the medium will diminish the number of colonies produced by TGF-β (23, 24). The interactions of these factors on the formation of colonies in soft agar have been studied extensively in recent years in systems using either growth factor-depleted medium or bovine plasma instead of fetal bovine serum. The addition of 10% fetal bovine serum to the cultures does not change the sensitivity of the system to detect TGF-β in picogram concentrations but greatly reduces the capacity of additionally added PDGF and IGF to affect TGF-β-mediated colony growth. High concentrations of PDGF (10 ng/ml) do, however, increase colony numbers at constant TGF-β concentrations by 50% (26). We also observed synergistic effects between IL-1 at concentrations of 100 U/ml and TGF-β-containing medium on colony formation. PDGF has not yet been detected in conditioned medium from bone cultures and it is also unlikely that bone cultures produce such high concentrations of IL-1. However, interactions of TGF-β with other local growth factors produced by bone are possible and might enhance TGF-β activity on colony formation. Two local growth factors in bone distinct from TGF-β are bone-derived growth factor (BDGF) (12, 27) and skeletal growth factor (SGF) (28–30). Neither of these factors possesses TGF-β activity in the soft agar assay (12, 31), but it remains possible that they could modulate TGF-β promotion of colony formation.

We cannot tell from our experiments the mechanism of production or source of TGF-β in resorbing bone cultures. TGF-β is present in the conditional medium of most cell cultures and in platelets in a latent form and requires exogenous acid treatment for activation (32, 33). We did not add exogenous acid to our bone culture conditioned medium before assay for TGF-β activity. However, acid production by osteoclasts is an essential feature of the bone resorption process. We think it likely that TGF-β is released in latent form by bones incubated with resorbing agents and that acid production by osteoclasts or other bone cells leads to its activation, since we have found that when conditioned medium harvested from resorbing bones is acidified to pH 2, there is a marked increase in TGF-β activity in the medium.

A major issue that arises is the source of TGF-β in our bone culture system. Bone cells can synthesize TGF-β (34, 35), and TGF-β has been purified from demineralized bone matrix (13). It is difficult to assess whether TGF-β in the bone culture medium is present as a direct consequence of the resorption of the bone matrix or whether it represents an epiphenomenon associated with the effects of the hormones on bone cells to influence cellular release of TGF-β. PTH- and IL-1-treated cultures showed large differences in TGF-β activity for the same degree of bone resorption, and inhibition of increased bone resorption only partially inhibited the increase in TGF-β activity. It is therefore likely that most of the measured TGF-β activity is due to bone cell-derived TGF-β. However, the fact that there was a decrease in activity after CT treatment of the bone cultures suggests that at least part of the TGF-β activity is directly related to resorption.

Stimulatory and inhibitory effects of TGF-β on proliferation and differentiation in cell lines have been reported recently (36–40). Our results and those of others (15, 16) suggest that bone may also be an important target tissue for TGF-β. TGF-β stimulates DNA synthesis in fetal rat calvaria cultures and increases alkaline phosphatase activity, a marker for the osteoblastic phenotype, in the osteoblastic osteosarcoma cell line ROS 17/2.8 (17). Increases in TGF-β activity during bone resorption are likely to stimulate subsequent proliferation and differentiation of cells in the osteoblastic lineage. TGF-β might therefore contribute to the linkage of bone formation to previous bone resorption. This process is termed coupling and is a key feature of human adult bone remodeling in which it serves to regulate the balance between bone resorption and bone formation (14). Since coupling is a local site-specific phenomenon, it is likely to be regulated by local factors. In addition to TGF-β, these may include the already-mentioned SGF, a mitogenic protein of 11,000 daltons (28–30), and BDGF, also an 11,000-dalton protein, that, in addition to its mitogenic effects, stimulates collagen synthesis in fetal rat calvariae (12, 27). Both are apparently different from TGF-β. SGF secretion into conditioned medium from embryonic chicken bones was reported to be enhanced by PTH (30). How these factors and TGF-β interact and what precise function each of these local growth factors fulfills has yet to be determined.

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