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# TRANSFORMING GROWTH FACTOR-BETA INHIBITION OF MINERALIZATION BY NEONATAL RAT OSTEOBLASTS IN MONOLAYER AND COLLAGEN GEL CULTURE

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## SUMMARY

The latent form of transforming growth factor-beta (TGF-$\beta$) is a component of the extracellular matrix of bone. The active form, when locally injected in vivo, stimulates both inflammation and ectopic bone formation. The present study was undertaken to determine if TGF-$\beta$ also stimulated mineralization by isolated rat calvarial osteoblasts cultured in collagen gels. Gels were used because they should mimic in vivo conditions better than classical monolayer culture. Compared to cells in monolayers, osteoblasts cultured in collagen gels exhibited slower growth, but higher alkaline phosphatase activity and mineral deposition. Cultured cells also synthesized the osteoblast-specific marker, osteocalcin. The increase in osteocalcin in cell layers was parallel to the increase in mineral deposition. In the presence of TGF-$\beta$, neither cell growth nor alkaline phosphatase activity increased. Instead, a small decrease occurred in both parameters when compared to untreated cultures. Accumulation of collagen, the major component of the extracellular matrix where mineralization occurs, was similar in untreated and TGF-$\beta$1-treated cultures. However, 8 p*M* TGF-$\beta$1 dramatically suppressed mineral deposition in both types of cultures. Despite TGF-$\beta$1 stimulating a fourfold increase in lactic acid, the consequent increase in culture medium acidity did not account for the inhibitory effects of TGF-$\beta$1 on mineralization. These results demonstrate that collagen gel culture is an improved technique over conventional monolayer culture for demonstrating differentiated osteoblast function and sensitivity to TGF-$\beta$1. TGF-$\beta$1, at a concentration that has little effect on cell growth, alkaline phosphatase activity, or collagen accumulation, is a potent inhibitor of mineralization. The mechanism by which TGF-$\beta$1 inhibits mineralization remains to be determined.

*Key words:* osteoblasts; bone; collagen gels; mineralization; TGF-beta.

## INTRODUCTION

Transforming growth factor-betas (TGF-$\beta$s), a family of closely related polypeptides, exert a wide variety of effects on many types of cells (10,30,45). The effects include regulation of extracellular matrix synthesis and degradation, as well as cell proliferation and differentiation. First discovered and named because of its ability to stimulate untransformed fibroblast growth in semisolid medium (39), TGF-$\beta$ is found primarily in blood platelets and bone matrix (2,43). Neither the cellular mechanism(s) of action of TGF-$\beta$ nor the physiological trigger for activation of its latent form are known. Activation of TGF-$\beta$ appears to occur either on the cell membrane or extracellularly. Osteoclasts can participate as well as secreted proteins (e.g., plasmin, transglutaminase, and thrombospondin) (26,36,41). A larger group of proteins, called the TGF-$\beta$ superfamily, have ca. 30% sequence identity to TGF-$\beta$1 and include the bone morphogenetic proteins (BMPs), which stimulate endochondral ossification. Activated TGF-$\beta$s bind to several types of high affinity receptors on bone cell membranes (31), but the signal transduction mechanism(s) for the TGF-$\beta$ receptors have yet to be elucidated. Pharmacological levels of TGF-$\beta$ locally administered in vivo to calvaria of neonatal rats resulted in a doubling of the thickness of the skeletal tissue (33,47), but the change may have been secondary to other inflammatory-like alterations noted (i.e., increased vascularity, fibrosis, and/or the presence of osteoclasts that normally are absent at this developmental stage).

The effects of TGF-$\beta$1 in culture depend on cell type, growth stage, and cellular environment. Proliferation of endothelial cells was reported to be inhibited by TGF-$\beta$ in monolayer but not in collagen gel culture (29). In osteoblastic cell lines, growth was inhibited by TGF-$\beta$ (10), but altered in a biphasic manner in osteoblast-enriched, osteoprogenitor cell cultures (9). In rabbit articular chondrocyte cultures, TGF-$\beta$1 inhibited proliferation in low serum, increased growth in high serum, and different receptor systems for TGF-$\beta$1 were expressed under each serum condition during the cell cycle (51). The ability of TGF-$\beta$ to increase extracellular matrix components, either by stimulating synthesis or decreasing degradation, has been reported both for cells from nonbone (38) and skeletal tissues (9,10,24). Matrix changes, potentially able to modify cell shape, have been suggested as a mechanism for TGF-$\beta$ effects on cell proliferation and differentiation (15,38). However, TGF-$\beta$ stimulation of cultured chondrocytes to become flattened and fibroblastic was not associated with a change in the differentiated proteoglycan phenotype of the matrix (23). On the other hand, changes in other matrix components (e.g., collagen, fibronectin, etc.) may be more important than proteoglycans. In serum-deprived confluent cultures of osteoblast-enriched cells from fetal rat parietal bones, 40–400 p*M* TGF-$\beta$1 treatment for 1–2 d resulted in a 2-fold increase in mRNA encoding the $\alpha$1-chain of type I collagen, a 3-fold increase both in total (medium plus cell layer) collagen and noncollagen accumulation, as well as a 10-fold increase in collagen asso-

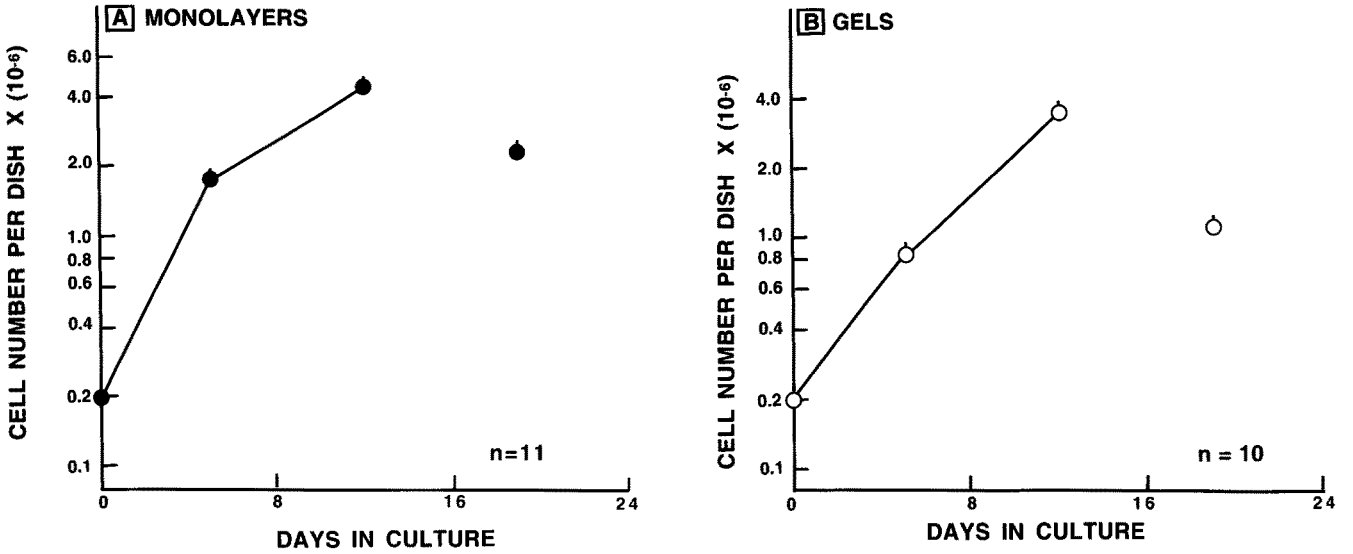


FIG. 1. Growth of osteoblasts in (*A*) monolayer and (*B*) collagen gel cultures. Values are mean ± SEM of 11 determinations in 5 experiments.

ciated with the cell layer (8). Although 4 and 40 p*M* TGF-β1 also increased DNA content by 2.5–3.5-fold in 2 d , 400 p*M* TGF-β1 had no effect on DNA content. In chondrocytes, TGF-β stimulated fibronectin production in vitro (40,54) and addition of fibronectin to cultured chondroblasts inhibited cellular differentiation (40). However, other mechanisms besides alterations in synthesis of matrix components also have been proposed to explain the actions of TGF-β (35,42,50).

The present study was undertaken to determine if culture of osteoblasts in collagen gels, which create a more tissuelike environment, results either in improved ability of cells to mineralize or earlier onset of mineralization compared to culture in monolayers. In addition, the effect of TGF-β on mineralization by cultured osteoblasts was investigated.

## MATERIALS AND METHODS

*Materials.* Porcine platelet TGF-β1 (96% pure) was purchased from R & D Systems (Minneapolis, MN). Human recombinant TGF-β1 was provided gratis by Dr. Edward Amento of Genentech Co. (San Francisco, CA). Crude collagenase (CLS 2) was from Boehringer Mannheim Co. (Indianapolis, IN). Media and other reagents for cell culture were from GIBCO (Grand Island, NY). Porcine insulin was provided gratis by Dr. M. Niedenthal, Lilly Research Laboratories (Indianapolis, IN). Dr. M. Uskokovic, Hoffman-LaRoche (Nutley, NJ), supplied gratis 1,25-dihydroxycholecalciferol. Kits for radioimmunoassay of osteocalcin were purchased from Biomedical Technologies (Stoughton, MA).

*Isolation and culture of cells.* Osteoblastlike cells were isolated from calvaria of Sprague-Dawley neonatal (1-wk-old) rats (Sasco King Labs, Oregon, WI). Parietal bones of calvaria were washed in phosphate-buffered saline (PBS), cells dissociated with 0.1% crude collagenase (Worthington Biochemicals, Freehold, NJ), washed and osteoblasts obtained between the first and second hours of enzyme treatment (53) either embedded in collagen gels or used to inoculate monolayer cultures. Cells were inoculated at $0.2 \times 10^6$ cells in 35-mm dishes (Falcon, Oxnard, CA), cultured in Biggers Gwatkin Judah-Fitton-Jackson modification (BGJ-FJ) medium supplemented with 10% fetal calf serum (FCS), 50 μg/ml Gentamicin, and 25 m*M* HEPES buffer (GIBCO). Routinely, fresh medium with 10 m*M* β-glycerophosphate was added on Day 1 and medium changed each 2–3 d. Some cultures on Day 1 also received TGF-β1. Cultures were incubated at 37° C in a humidified atmosphere of 5% $CO_2$ in air. Replicate cultures for each condition studied were assayed at weekly intervals during culture in each of two to five experiments.

*Culture of cells in collagen gels.* Vitrogen 100 (Collagen Corp., Palo Alto, CA) was mixed with a fivefold concentrate of Ham's F12 culture medium and 0.2 *N* NaOH, (i.e., 1:0.28:0.06, vol/vol/vol), to bring the final mixture to pH 7.4 and a physiologic salt concentration. A 50 μl aliquot of calvarial cell suspension containing $0.2 \times 10^6$ cells was added to 1.0 ml of the solution and the mixture incubated 5 min in a 37° C water bath to form solid gels. Dishes containing solidified gels with embedded cells then were overlaid with culture medium.

*Quantitation of cells.* At weekly intervals over a 19-d period, collagen gels containing cells or cells scraped from monolayers were homogenized and aliquots mixed with 0.1 *M* citric acid–0.1% crystal violet for nuclear enumeration by microscopic hemacytometer counting as described previously (46). The validity of this method was confirmed by comparing numbers of cells using this technique with whole cells released from monolayers by trypsinization. Cell counts were determined per culture dish.

*Measurement of collagen accumulation.* Following removal of medium and rinse of cultures with PBS, monolayers were hydrolyzed in 6 *N* HCl at 105° C overnight. Proline and hydroxyproline in dried hydrolysates were derivatized with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Sigma Chemical Co., St. Louis, MO). The derivatization mixture contained hydrolysate dissolved in water (0.1 ml); 0.4 *M* potassium tetraborate buffer, pH 9.5 (0.1 ml); and 2 m*M* 7-chloro-4-nitrobenz-2-oxa-1,3-diazole in ethanol (0.1 ml). The mixture was incubated in the dark at 37° C, 20 min, and the reaction stopped by adding 0.05 ml 1 *N* HCl and 0.15 ml methanol/water (1:1, vol/vol). Thin-layer chromatography of an aliquot of the diluted mixture was used to separate hydroxyproline ($R_f$, 0.23) from proline($R_f$, 0.57). After spotting of the mixture on 20 × 20 cm silica gel-coated plates (Sigma), plates were chromatographed in a solvent of chloroform/triethylamine/methanol (8:1:1, vol/vol/vol) (1). Hydroxyproline spots from samples and standards were scraped, eluted in methanol/water (1:1, vol/vol), and quantitated at 495 nm using a Beckman 25 spectrophotometer. The amount of hydroxyproline in samples was multiplied by a factor of 10 to calculate level of collagen (5).

*Assay of alkaline phosphatase activity.* Cells in monolayers or collagen gels were rinsed with balanced salt solution, homogenized at 4° C in 1% Triton X-100 using a Potter-Elvehjem homogenizer and the resultant homogenates used to assay enzyme activity. Alkaline phosphatase was measured by colorimetric assay of hydrolysis of ρ-nitrophenylphosphate (28). The assay mixture contained 0.5 ml homogenate in 1% Triton X-100; 0.1 ml 1.5 *M* 2-amino-2-methyl-1-propanol buffer, pH 10.3; 0.01 ml 0.43 *M* $MgCl_2$; and 0.07 ml 50 m*M* ρ-nitrophenylphosphate (Sigma). Following

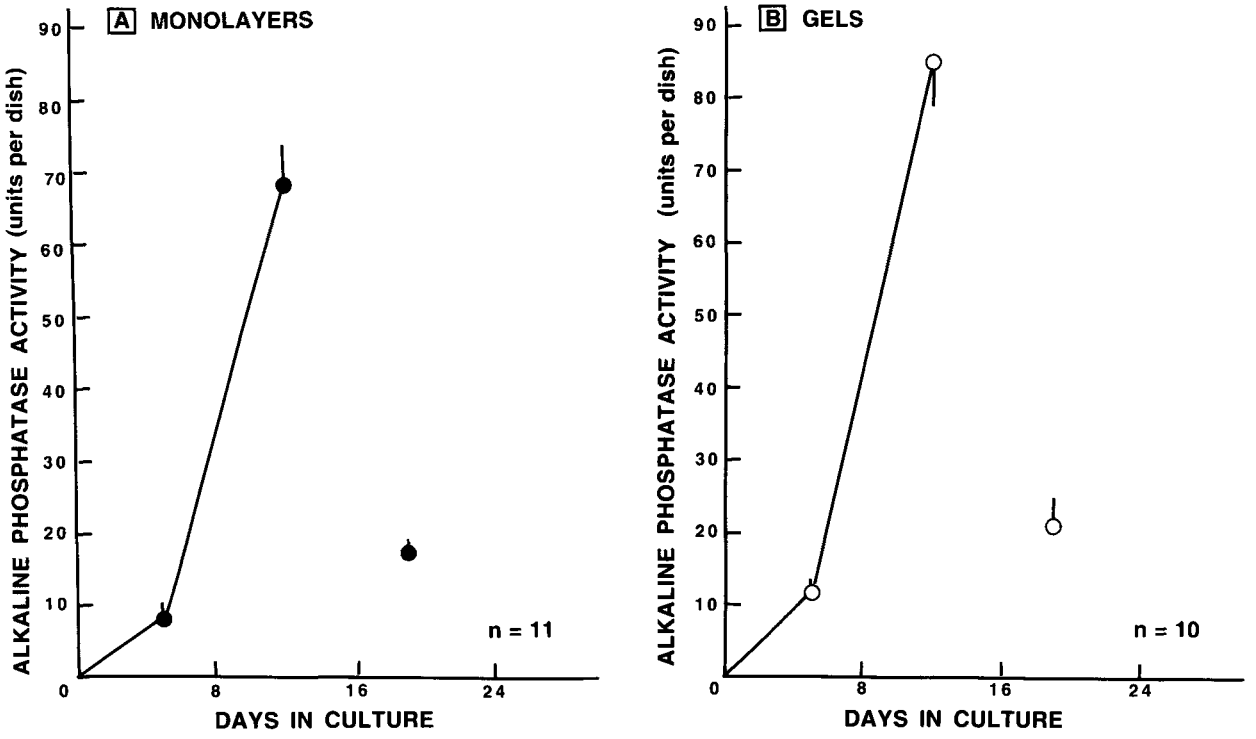


FIG. 2. Alkaline phosphatase activity in (*A*) monolayer and (*B*) collagen gel cultures. Values are mean ± SEM of 11 determinations in 5 experiments.

incubation at 37° C, 15 min, the reaction was stopped by adding 3 ml 0.15 *N* NaOH and absorbance measured at 410 nm. One unit of enzyme activity was defined as an absorbance change of 1.0, which resulted in formation of 0.216 μmoles of *p*-nitrophenol per 15 min at 37° C.

*Quantitation of mineralization.* Aliquots of cellular homogenates from monolayers or collagen gels were placed in porcelain crucibles, dried overnight at 110° C, and then ashed overnight in a muffle furnace at 650° C. Ash was dissolved in concentrated hydrochloric acid (HCl) and diluted to 1 *M* HCl, 1% lanthanum chloride. Calcium (μmoles) was quantitated by atomic absorption spectrometry using a Perkin-Elmer 460 instrument and calcium standards.

*Radioimmunoassay of osteocalcin.* To extract osteocalcin from gel cultures, the method of Gundberg et al. (17) was used. After medium was removed for separate assay, gels were rinsed with balanced salt solution, ion-free water, and then frozen at −70° C. Following lyophilization, samples were extracted 16 h at 4° C in a cocktail containing 0.5 *M* ammonium ethylenediaminetetraacetic acid (EDTA), pH 6.2, 30 μ*M* phenylmethylsulfonyl fluoride, 100 μ*M* *p*-hydroxymercuribenzoic acid, 10 m*M* 6-aminocaproic acid, and 5 m*M* benzamidine. Extracts then were measured for osteocalcin with a nonequilibrium radioimmunoassay described by Lian et al. (27) and a commercial kit obtained from Biomedical Technologies. Assay buffer included 0.122 *M* NaCl, 0.01 M $NaH_2PO_4$, 0.025 *M* $Na_4EDTA$,

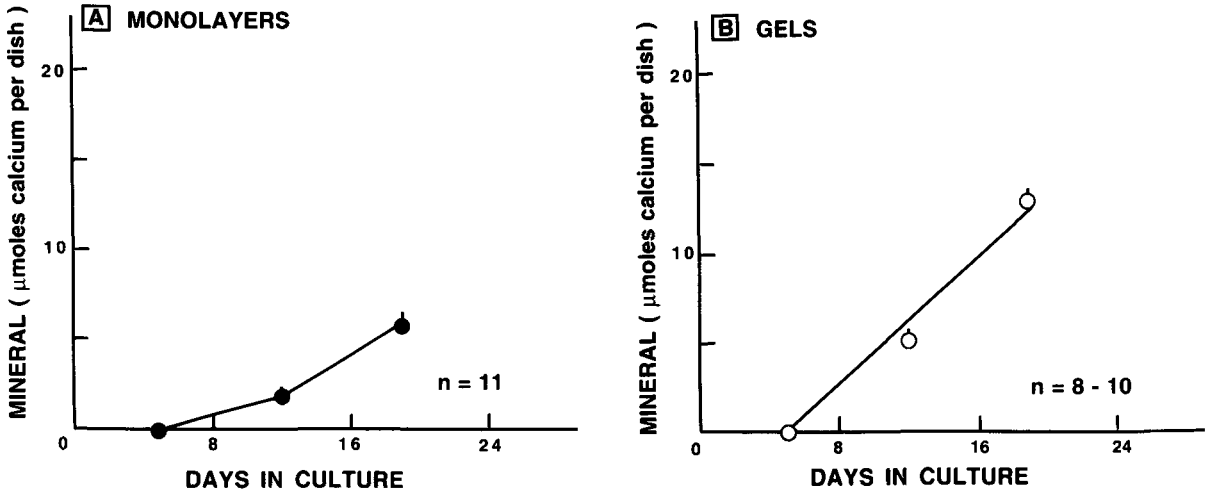


FIG. 3. Mineral deposition by osteoblasts in (*A*) monolayer and (*B*) collagen gel cultures. Values are mean ± SEM of 11 determinations in 5 experiments.

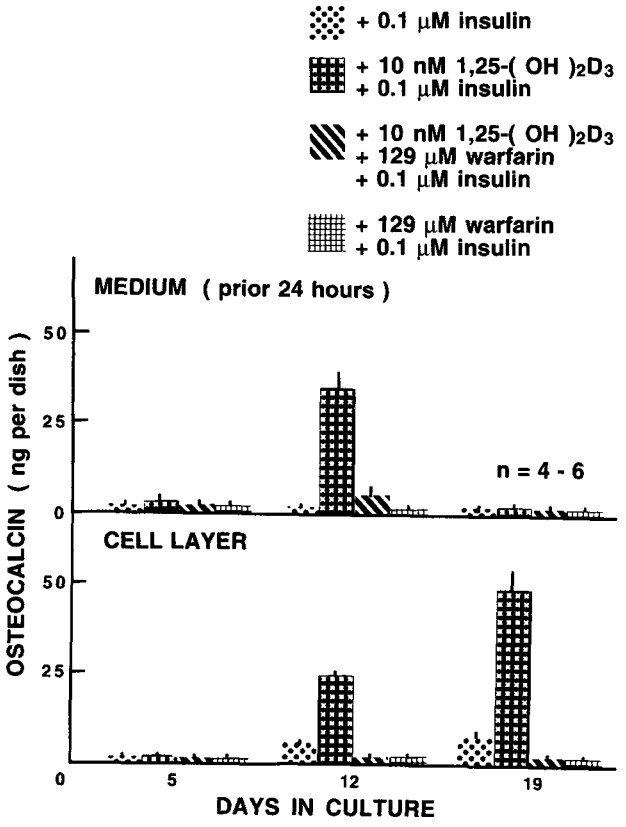


FIG. 4. Osteocalcin in medium (prior 24 h) or cell layer (cumulative) in collagen gel cultures. Osteoblasts were cultured in medium containing 0.1 μ*M* insulin alone (*dotted bar*) or with insulin and 10 n*M* 1,25-dihydroxycholecalciferol (*hounds tooth bar*), 10 n*M* 1,25-dihydroxycholecalciferol plus 129 μ*M* warfarin (*wide diagonals bar*), or 129 μ*M* warfarin (*cross hatched bar*). Values are mean ± SEM of three to six determinations in two experiments.

0.1% (wt/vol) Tween 20, 0.1 (wt/vol) bovine serum albumin (BSA), pH 7.4. Each assay contained 0.2 ml buffer containing rat osteocalcin standard, medium, or gel extract (0–40 μl) and 0.1 ml buffer with goat anti-rat osteocalcin and normal goat serum. After incubation for 16 h at 4° C, 0.1 ml buffer containing 1 μCi $^{125}$I-rat osteocalcin was added and incubation continued for 16 h at 4° C. Assay tubes then received 0.1 ml buffer containing donkey anti-goat gamma-globulin and, following incubation for 2 h at 4° C, tubes were centrifuged, supernates discarded, and pellets counted in a Beckman 7000 gamma counter. Osteocalcin from gel extracts or culture medium was quantitated from the rat osteocalcin standard curve and expressed per culture dish. The standard curve was linear from 0.03–1.0 ng per tube. Sample dilutions were varied to verify linearity.

*Lactic acid determination.* Lactic acid in culture medium was measured as described by Nichols and Neuman (32). Following deproteinization of medium with 8% perchloric acid, samples were centrifuged and supernates used for assay. The quantitative conversion of lactate to pyruvate by lactate dehydrogenase (Sigma) was coupled to nicotinamide adenine dinucleotide (NAD) reduction and measured spectrophotometrically at 340 nm as described by Sigma Technical Bulletin 826-UV. Lactic acid levels were determined from a lactic acid standard curve and expressed per culture dish. The assay was sensitive to 2 μg per tube.

*Statistical analysis.* Replicate cultures from each of two to five separate cell isolations were measured, means and standard errors determined, and significance of difference between treatment and control groups was tested with the Student's *t* test.

## RESULTS

*Cell growth.* To determine if culture of osteoblasts in a collagen matrix resulted in reduced cell growth but increased function, a comparison was made of osteoblast growth in collagen gel and monolayer culture. Although the pattern of growth was similar in the two types of cultures, a slower growth rate occurred in collagen gels resulting in a lower overall cell density when confluence was reached on Day 12 (Fig. 1). At confluence (i.e., highest cell number obtained per dish), gels contained osteoblasts in clusters with the microscopic appearance of regional confluence throughout the gel. In both types of cultures, a decrease in cell number was seen on Day 19 of culture. The greater decrease in osteoblast number on Day 19 in collagen gel cultures may have been a consequence of increased cell function and terminal differentiation.

*Onset of differentiation.* To ascertain whether differentiated osteoblastic characteristics (e.g., elevated alkaline phosphatase activity, mineral deposition, and synthesis of osteocalcin) were present when cells were cultured in collagen gels, these features were measured and compared when appropriate with cell functions in monolayer culture. To permit data to be calculated per cell as well as expressed per dish, cell numbers were measured and mean values used to calculate activity or level per cell. Although the pattern of change in alkaline phosphatase activity was similar in collagen gel and monolayer cultures, activity per cell was higher in gel cultures. Activity in both types of cultures was low on both Days 5 and 19 of culture, but peaked on Day 12 (Fig. 2) when confluence was reached and onset of mineral deposition occurred. By Day 12 of culture, units of alkaline phosphatase activity per million cells was 24.7 in collagen gels but only 15.1 in monolayer cultures.

In neither type of culture was mineral deposition measurable until the second week of culture. On Day 12, mineral deposited in monolayer cultures barely was measurable whereas in collagen gels, the level was threefold higher (Fig. 3). Mineral expressed per million cells was 1.46 in collagen gels but only 0.42 in monolayer cultures. No mineral deposition occurred in gels without cells or in gels containing a comparable number of isolated mouse embryo cells.

In collagen gel cultures, an increase in osteocalcin, a specific marker of mature osteoblasts, was parallel to the increase in mineral deposition in cell layers (Fig. 4). During the first 5 d of culture, osteocalcin levels in both gels and medium (Days 4–5) were below the limit of assay detection. By Day 12 of culture, osteocalcin was present both in culture medium as well as in cell layers. By Day 19, however, osteocalcin levels in the cell layer had doubled while that in culture medium was undetectable. Although osteocalcin was highest (i.e., fivefold higher) in the presence of 1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2D_3$] and insulin, a low level of osteocalcin was present in cell layers by Day 12 when only insulin was added to culture medium. Warfarin, a vitamin K antagonist of gamma-carboxyglutamyl synthesis, blocked the increase in osteocalcin in response both to 1,25-$(OH)_2D_3$ and insulin.

*Effect of TGF-β on osteoblast characteristics.* To test whether TGF-β altered growth or function of osteoblasts, cells were cultured in collagen gels or monolayers in the presence or absence of 1–10 p*M* TGF-β1. At concentrations of 8–10 p*M* TGF-β1, a slight but significant inhibition of growth of osteoblasts occurred in both collagen gel and monolayer cultures (Fig. 5). Cultures receiving 8 p*M* TGF-β1 contained 82–84% of the number of cells in untreated cultures on Day 12.

Osteoblasts cultured in collagen gels were more responsive to effects of TGF-β1 on alkaline phosphatase activity than cells cultured as monolayers (Fig. 6). There was little effect of 8 p*M* TGF-

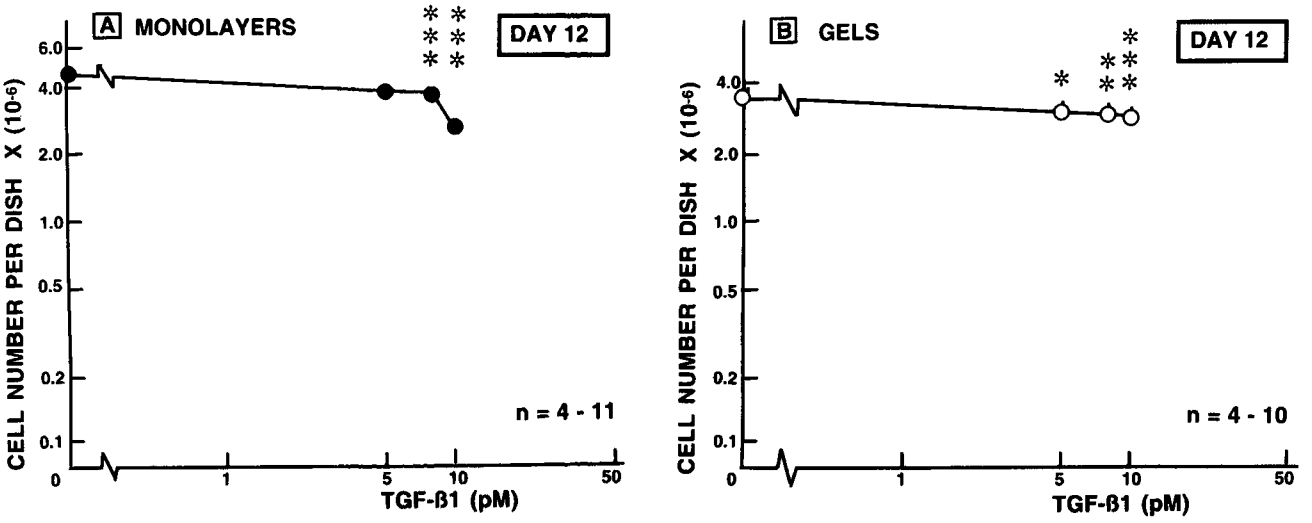


FIG. 5. Effect of varying concentrations of TGF-$\beta$1 on cell growth of osteoblasts in (*A*) monolayer and (*B*) collagen gel cultures. Values are mean ± SEM of 4–11 determinations in 5 experiments. ***, $P < 0.005$ vs. controls. **, $P < 0.01$ vs. controls. *, $P < 0.05$ vs. controls.

$\beta$1 on alkaline phosphatase activity in monolayer cultures, but activity in collagen gel cultures receiving TGF-$\beta$1 was only 68% of that in untreated gels. Alkaline phosphatase activity per million cells in untreated and TGF-$\beta$1-treated collagen gels cultures was 25 and 20 units, respectively. In contrast, activity in both treated and untreated monolayer cultures was 15–17 units per million cells.

Even though mineral deposition was much less in monolayer than in collagen gel culture, inhibition of mineralization by TGF-$\beta$1 was similar in both types of cultures. Both human recombinant and porcine platelet TGF-$\beta$1 were equally effective and half-maximal inhibition occurred with 4 p*M* TGF-$\beta$1 (Fig. 7). Near maximal inhibition of mineralization was seen with 8 p*M* TGF-$\beta$1, whereas 10 p*M* TGF-$\beta$1 also frequently was associated with extensive contraction of cell layer matrices in both collagen gel and monolayer cultures.

Inhibition of mineralization by TGF-$\beta$1 was not associated with suppression in cell layers of accumulation of collagen, the major component of the extracellular matrix surrounding osteoblasts (Fig. 8). Instead, collagen accumulation by Day 19 in cell layers of cultures treated with 8 p*M* TGF-$\beta$1 exhibited a slight but significant

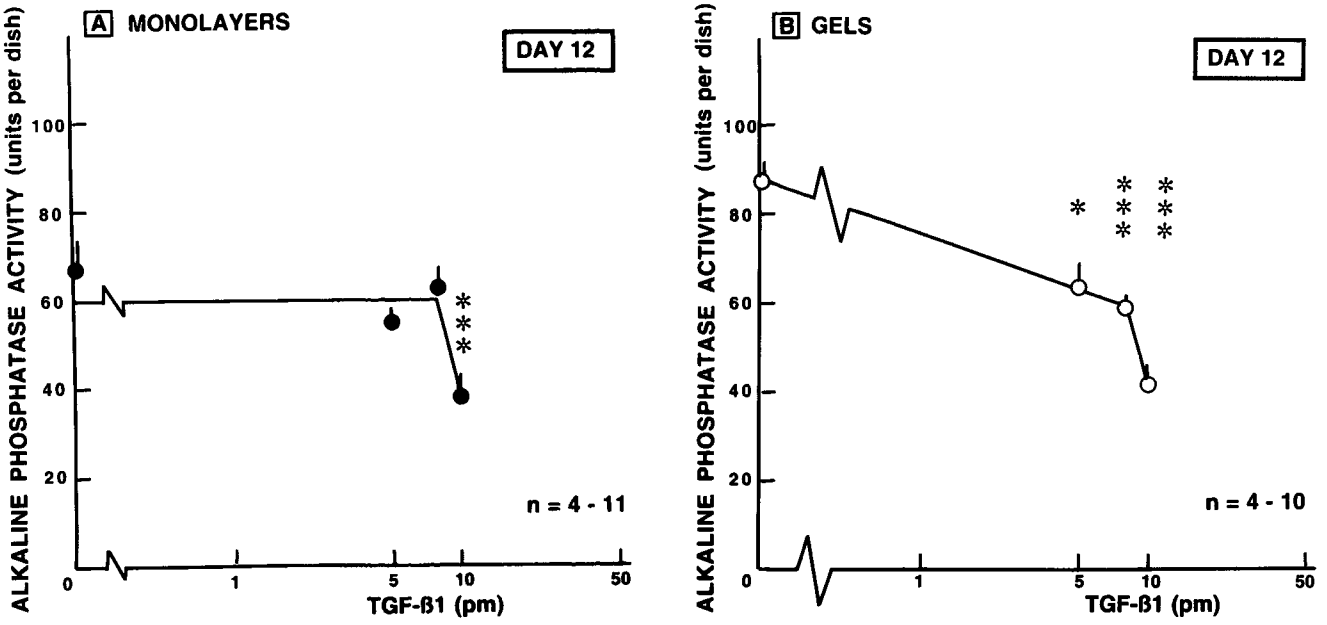


FIG. 6. Effect of varying concentrations of TGF-$\beta$1 on alkaline phosphatase activity in (*A*) monolayer and (*B*) collagen gel cultures. Values are mean ± SEM of 4–11 determinations in 5 experiments. ***, $P < 0.005$ vs. controls. *, $P < 0.05$ vs. controls.

increase relative to that in untreated cell layers. Even in untreated dishes, matrix deposition at Day 12 was adequate to permit cell layers to be detached as sheets from monolayer culture dishes. Cultures receiving TGF-β1 contained very little mineral at Day 19 and slightly higher cell numbers than those found in mineralized, untreated cultures. Consequently, when collagen in cell layers was expressed per million cells, collagen levels were similar in TGF-β1-treated and untreated cultures at Day 19 (i.e., 102 μg and 118 μg per million cells, respectively).

Because increased acid production frequently was observed by the second week in cultures treated with TGF-β1, the effect of TGF-β1 on lactic acid production also was measured. By Day 15 of culture, a fourfold increase in lactate in medium of TGF-β1-treated cultures relative to controls occurred (Fig. 9). To determine if increased acidification of medium in TGF-β1-treated cultures was the factor preventing mineral deposition, dilute hydrochloric acid was added to medium of control cultures to reproduce the rate of decrease in medium pH occurring in TGF-β1 treated cultures over a 15-d period. Addition of dilute acid did not block mineralization as observed in the presence of 8–10 p*M* TGF-β1. Consequently, TGF-β1 does not prevent mineralization by stimulating mildly acidic conditions (i.e., medium pH of 6.6–6.8 by day 15 of culture).

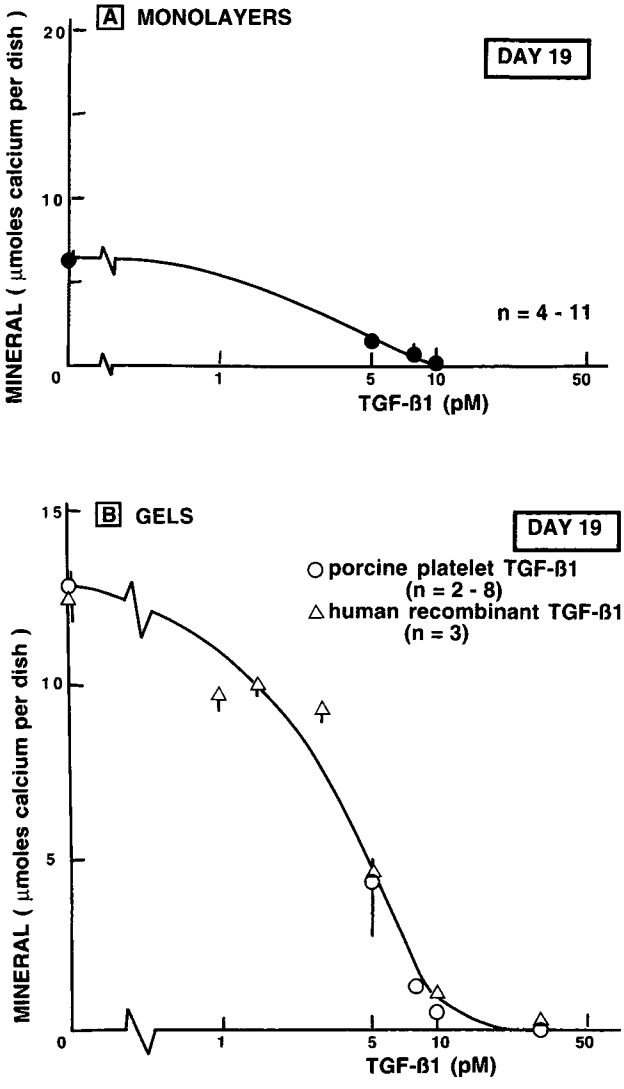


FIG. 7. Effect of varying concentrations of TGF-β1 on mineral deposition by osteoblasts in (*A*) monolayer and (*B*) collagen gel cultures. Values are mean ± SEM of 4–11 determinations in 5 experiments.

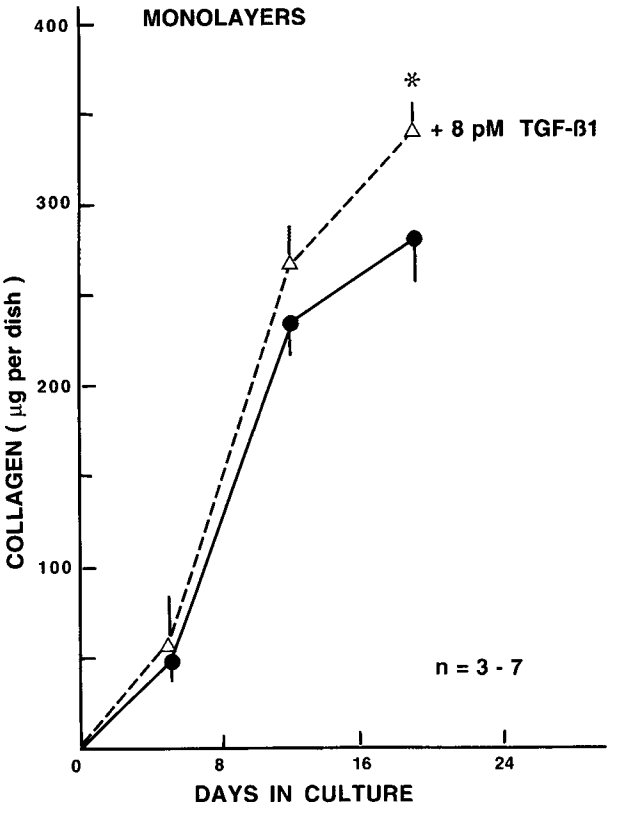


FIG. 8. Effect of 8 p*M* TGF-β1 (△) on collagen accumulation in cell layers of monolayer cultures. Values are mean ± SEM of three to seven determinations in two experiments. *, $P < 0.05$ vs. controls (●).

## DISCUSSION

The present studies indicate that culture of osteoblasts in collagen gels results in increased mineralization relative to conventional monolayer culture and provides an improved method to investigate differentiated osteoblast function and sensitivity to TGF-β. Although mineralization and responsiveness to TGF-β are increased when osteoblasts are cultured in collagen gels, it is not possible to decrease the time of onset of mineralization by culturing osteoblasts in the more tissuelike environment that collagen gels provide. In both monolayer and collagen gel culture, the earliest sign of mineralization occurs at Day 11. As occurs in vivo during bone formation, mineral deposition follows osteoblast secretion of a collagenous matrix. Besides collagen, bone matrices in vivo also contain low levels of other noncollagenous proteins (e.g., osteocalcin and the latent form of TGF-β). The precise functions of these proteins remain unknown. The appearance of osteocalcin in embryonic bone has been shown to coincide with mineral deposition and to increase with increasing bone density (20). Osteocalcin levels in osteoblast gel

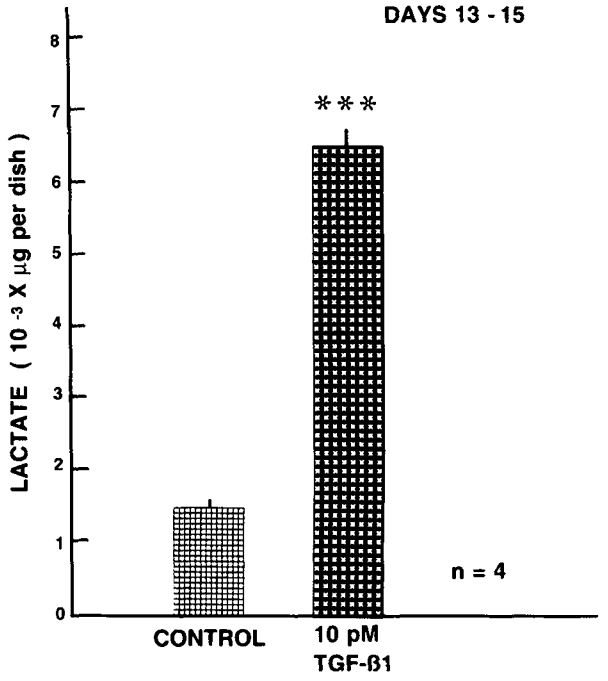


FIG. 9. Effect of 10 p*M* TGF-$\beta$1 on lactic acid accumulation in medium (Days 13–15) of collagen gel cultures. Values are mean ± SEM of four determinations from two experiments. ***, $P < 0.005$ vs. controls.

cultures in the present studies were comparable to those reported in 21-d fetal rat calvaria (i.e., 14.5 ± 0.5 ng osteocalcin per calvaria) (27). These investigators reported that addition of 0.1 $\mu M$ insulin to fetal rat calvaria in organ culture did not alter osteocalcin levels but enhanced the effect of 1,25-$(OH)_2D_3$. Although osteocalcin was stimulated maximally in the present studies in the presence of both insulin and 1,25-$(OH)_2D_3$, low levels of osteocalcin were present in cell layers of cultures receiving insulin but no 1,25-$(OH)_2D_3$.

In studies of fetal bovine osteoblasts in monolayer culture (21,52), mineralized matrices formed after 18–24 d and both cell number and alkaline phosphatase levels decreased as mineralization proceeded. Neither the precise role of alkaline phosphatase in the mineralization process nor the endogenous substrate used as a phosphate source during mineralization in vivo are known. Bellows et al. (4) using rat calvarial cells in monolayer culture reported that only the initiation phase of mineralization was dependent on alkaline phosphatase activity and the organophosphate added to support mineralization (i.e., $\beta$-glycerophosphate), largely was degraded in the process). Addition of 10 m*M* $\beta$-glycerophosphate to cultures of hypertrophic, but not nonhypertrophic chondrocytes from chick embryos, resulted in increased mRNA for type X collagen (11). This form of collagen is synthesized during hypertrophy and onset of mineralization.

In studies of TGF-$\beta$ response by dermal fibroblasts cultured in monolayer and collagen gels, TGF-$\beta$ increased collagen synthesis measured by $^3$H-proline incorporation and collagenase digestion similarly in each type of culture (16). In the present studies, the effect of TGF-$\beta$ on total collagen accumulation in cell layers of monolayer cultures was a small increase in collagen per dish, but not per cell. Total collagen accumulation was measured in order to circumvent potential problems of TGF-$\beta$ effects on cellular uptake of $^3$H-proline, as well as the possibility that mineral deposition in matrices might limit susceptibility to collagenase. Inhibition of alkaline phosphatase activity by TGF-$\beta$ generally has been observed in primary osteoblast-enriched cultures, as well as in cultures of chondrocytes and osteoblast cell lines (10,12,34), but with some exceptions (7,37). Chondrocyte mineralization was inhibited by TGF-$\beta$ when cells were cultured as a pellet (25). More recently, chick embryo sternal chondrocytes receiving TGF-$\beta$ in agarose culture also were shown to exhibit loss of differentiated properties (49). These observations contrast with those in vivo, where TGF-$\beta$ was reported to increase intramembranous bone formation (3,47). However, effects of TGF-$\beta$ in vivo may be largely a consequence of its action on nonosteoblastic cells. Osteoclasts have been shown to activate latent TGF-$\beta$ by an unknown mechanism (36), and bone resorption to be stimulated by TGF-$\beta$ (48). Recently, Harris et al. (18), studying fetal rat calvarial osteoblasts, reported that TGF-$\beta$ at 40–200 p*M* increased proliferation, but completely inhibited mineralized nodule formation, as well as expression of BMP-2, BMP-6, alkaline phosphatase, and osteocalcin. These investigators also showed that, while TGF-$\beta$ enhanced proliferation, but reversibly inhibited differentiation, BMP-2, at 20–50 ng per ml, enhanced differentiation and mineralized nodule formation by osteoblasts. In nonbone cells as well, TGF-$\beta$ inhibits differentiation (13,14,44). Actions of TGF-$\beta$ on nonbone cells are diverse and include basic metabolic changes (e.g., stimulation of glucose uptake and glycolysis) (6,22). The increased lactate production, in response to TGF-$\beta$ in the present studies, is consistent with stimulation of glycolysis.

The significance of inhibition of mineralization by TGF-$\beta$ is unknown, but could provide negative control on osteoblastic function. TGF-$\beta$ either could shift differentiated osteoblasts to a less differentiated state or prevent precursor cells from differentiating and mineralizing. Bone morphogenetic proteins, expressed by cultured fetal rat calvarial osteoblasts, were suggested to have specific roles in differentiation and mineralization (19). The ability of TGF-$\beta$ to block the expression of bone morphogenetic proteins may provide a mechanism for inhibition of mineralization by TGF-$\beta$. Low release and activation of latent TGF-$\beta$ stored in bone matrices may contribute to initial events in resorption during normal bone remodeling. However, release of activated TGF-$\beta$ could become excessive in bone diseases with impaired mineralization. Future studies are needed to determine if other isoforms of TGF-$\beta$ also inhibit mineralization, if osteocalcin production is altered by TGF-$\beta$, or if addition of bone morphogenetic proteins to cultured osteoblasts overcomes the inhibitory effects of TGF-$\beta$ on mineralization.

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