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Cell Biology

Stimulation of the expression of osteogenic and chondrogenic phenotypes *in vitro* by osteogenin

(alkaline phosphatase/osteoblasts/chondrocytes/periosteal cells/bone marrow stroma)

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ABSTRACT **Osteogenin was recently purified and the amino acid sequences of tryptic peptides were determined. Osteogenin in conjunction with insoluble collagenous bone matrix induces cartilage and bone formation *in vivo*. To understand the mechanism of action of osteogenin, we examined its influence on periosteal cells, osteoblasts, fibroblasts, chondrocytes, and bone marrow stromal cells *in vitro*. Osteogenin stimulated alkaline phosphatase activity and collagen synthesis in periosteal cells. The cAMP response to parathyroid hormone in periosteal cells was increased by osteogenin. In primary cultures of calvarial osteoblasts, osteogenin stimulated alkaline phosphatase activity, the cAMP response to parathyroid hormone, and the synthesis of collagenous and noncollagenous proteins; however, cell proliferation was not affected. Osteogenin increased the production of sulfated proteoglycans in fetal rat chondroblasts and in rabbit articular chondrocytes. The present experiments demonstrate the significant influence of osteogenin in the stimulation of osteogenic and chondrogenic phenotypes *in vitro*.**

Bone has a considerable potential for regeneration. However, the cellular and molecular mechanisms of bone regeneration are not clear. The presence of growth and differentiation factors in bone has been demonstrated by implantation of demineralized diaphyseal bone matrix in intramuscular and subcutaneous sites and results in local cartilage and bone formation (1, 2). The developmental cascade involves the following sequential steps: chemotaxis and attachment of mesenchymal stem cells, proliferation of progenitor cells, and differentiation of cartilage, bone, and hematopoietic marrow (3–5). Osteogenin, a bone-inductive protein, was isolated by heparin affinity chromatography (6). It was further purified and the amino acid sequences of tryptic peptides were determined and found to be unique (7). The amino acid sequence of bovine osteogenin (7) was similar to the amino acid sequence deduced from the cDNA clones of one of the human bone morphogenetic proteins, bone morphogenetic protein 3 (8). Whereas recombinant human bone morphogenetic proteins induced only cartilage (8), osteogenin purified from bovine bone matrix induces cartilage and bone formation *in vivo* (7, 9). To understand the mechanism of action of osteogenin, we have examined its influence on periosteal cells, osteoblasts, fibroblasts, chondrocytes, and bone marrow stromal cells *in vitro*. We report that osteogenin stimulates the expression of the osteogenic phenotype in periosteal cells, osteoblasts, and bone marrow stromal cells and of the chondrogenic phenotype in chondroblasts.

MATERIALS AND METHODS

Preparation of Purified Osteogenin. Osteogenin was purified as described (7). The method utilized extraction of demineralized bovine bone matrix with 6 M urea and purification by hydroxyapatite, heparin-Sepharose affinity chromatography, and molecular sieve chromatography on Sephacryl S-200. The most active fractions were further purified by preparative SDS/PAGE under nonreducing conditions and electroelution. Bone-inductive activity was localized between 30 and 38 kDa. SDS/PAGE under reducing conditions revealed a broad single band with a molecular mass of 22 kDa (7). Gel-eluted material from three lots was used. Each lot of osteogenin was assayed *in vivo* to determine the bone-inductive activity. The amount of active protein was estimated by the relative intensity of the silver-stained band of bovine osteogenin after SDS/PAGE under reducing conditions compared with bovine serum albumin (BSA) (Pharmacia) on the same gel. After gel elution, the protein was precipitated with ethanol at 4°C overnight; the pellet was then washed twice with 85% (vol/vol) ethanol, dried, and redissolved in 5 mM HCl/0.1% BSA. The stock preparations of osteogenin were frozen at −70°C in aliquots. The aliquots were thawed and dissolved in the appropriate medium for the experiment. As the qualitative *in vitro* responses to osteogenin were comparable for different lots, representative results are presented. Because a limited homology of bone morphogenetic proteins with transforming growth factor β (TGF-β) has been reported (8), experiments were performed with TGF-β type 1 (TGF-β_1) (R & D Systems, Minneapolis) for comparison. In some experiments a neutralizing antibody to TGF-β was used (R & D Systems). The antibody was added to the appropriate antigen 1 hr prior to the experiment. All cells studied were continuously exposed to osteogenin or TGF-β_1 with daily medium changes, unless specified otherwise.

Cultured Cells. *Osteoblasts and periosteal cells from fetal rat calvariae were prepared as described (10).* Parietal bones of 19- to 20-day rat (Fischer 344) fetuses were stripped of periosteum, dissected free of frontal and occipital bones and sutures, minced into 1- to 2-mm pieces, and digested at 37°C with bacterial collagenase type II (3 mg/ml; 163 units/mg; lot W8H415, Worthington) containing clostripain inhibitor (11, 12). Cell populations released after the first 15 min were discarded and, thereafter, the six successive 10-min digestions were combined. Cells were plated at 25,000 cells per cm^2 and grown to confluence in 24-well plates (Costar) in humidified 5% CO_2/95% air at 37°C. The culture medium was modified Ham's F-12 medium (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 20 mM Hepes (pH 7.4), (Biofluids, Rockville, MD), 10% (vol/vol) fetal bovine serum

Abbreviations: PTH, parathyroid hormone; TGF-β, transforming growth factor β; FBS, fetal bovine serum; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; CDP, collagenase-digestible protein; NCP, noncollagenous protein.

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(FBS) (GIBCO), 1% ITS+TM premix (Collaborative Research) [containing insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenious acid (6.25 μg/ml), BSA (1.25 mg/ml), and linoleic acid (5.35 μg/ml)], ascorbic acid (50 μg/ml; Sigma), gentamicin sulfate (1 μg/ml; GIBCO), and fungizone (0.25 μg/ml; Flow Laboratory). At confluence the medium was replaced with fresh medium containing 0.3% FBS, 0.1% BSA, 0.2% ITS, and osteogenin or TGF-β_1 as indicated. The cells were further cultured as indicated. For proliferation studies, cells were grown in 25-cm^2 plastic flasks (Nunc, Denmark) for 24–48 hr and then plated at 10,000 cells per well in 24-well plates.

The periosteal cell population was prepared from periostea obtained from parietal bones and then treated with collagenase type II (1.5 mg/ml; Worthington) (13). The cells from the first 15-min digestion were discarded, and the following four successive 15-min digestions were combined and cells were seeded in 24-well plates or grown in 25-cm^2 flasks in M-199 medium buffered with 25 mM Hepes (pH 7.4) (Biofluids), supplemented with 10% FBS, 1% ITS+TM Premix, and antibiotics, as described for osteoblasts.

Rat bone marrow stromal cell preparations. Fischer male rats (3–4 months old) were used. Marrow cells were flushed from the mid-shafts of the femur with a syringe containing serum-free medium, and a single-cell suspension was obtained by drawing the cells successively through 19-, 23-, and 25-gauge needles (14). About 2.5 × 10^7 cells per rat femur were obtained. The cells were plated in 25-cm^2 plastic flasks (Nunc) at 2.5 × 10^6 cells per flask in the same medium as described for osteoblasts from fetal rat calvariae. This results in the growth of clones of stromal cells (15, 16). The medium was first changed 5 days after plating, and every 3 days thereafter. Cells were incubated at 37°C in humidified 95% air/5% CO_2.

Fetal rat chondroblasts. Femurs of 20- to 21-day rat fetuses were stripped of perichondrium-periosteum and the nonvascularized cartilage region was dissected out and digested for three 20-min periods with collagenase type II (3 mg/ml; Worthington). Cells were pooled and plated at 50,000 cells in a 20-μl drop (17). After 60 min for cell attachment, the dishes were flooded with 0.5 ml of the same medium as used for calvarial osteoblasts.

Rabbit articular chondrocytes. Cartilage was dissected from the knee joints of 2-month-old New Zealand White rabbits. For each experiment, tissue was harvested from a single animal. The cartilage was minced, washed in serum-free Hanks' balanced salt solution (Biofluids) with antibiotics, and digested in collagenase type II (3 mg/ml; Worthington) for 2 hr. Cells released after each 30-min digestion were collected and fresh collagenase was added. Cells were pooled and plated at 50,000 cells in 20 μl per well in a micromass culture as described for fetal rat chondroblasts.

DNA Synthesis. DNA synthesis was determined by incorporation of [^{3}H]thymidine. Total DNA content was also determined. In some experiments 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Chemicon) colorimetric assay was used to estimate cell growth.

DNA labeling. Cells were incubated with [*methyl*-^{3}H]thymidine (5 μCi/ml; 247.9 GBq/mmol, NEN) in medium containing 0.5% FBS. At the end of a 4-hr incubation, the radioactive medium was aspirated, and ice-cold 10% (wt/vol) trichloroacetic acid containing 1 mM nonradioactive thymidine was added for 30 min. The cells were scraped from the culture wells and DNA was precipitated with 100 μg of BSA as a carrier. After centrifugation, the supernatant was discarded, and the acid-insoluble pellet was incubated at 90°C for 20 min in 0.5 ml of 10% trichloroacetic acid to hydrolyze DNA.

DNA content. DNA content was determined using bisbenzimidazole (Hoechst 33258) (18, 19). Briefly, from each well an 80-μl aliquot of homogenized cell sample was mixed with 1910 μl of phosphate saline buffer (0.05 M Na_2HPO_4/2.0 M NaCl/2 mM EDTA, pH 7.4) and 10 μl of a 20 mg % of bisbenzimidazole stock solution in H_2O. All standard curves were constructed with the same DNA standard stock solution frozen at −70°C (19).

Colorimetric assay for cell proliferation. MTT is cleaved by mitochondria to yield a dark blue formazan product. MTT was added to cultures during the last 4 hr of the experiment. Formazan was dissolved in isopropanol, containing 0.04 M HCl, giving a homogeneous blue solution and the absorbance was read within 1 hr at 570 nm (20). Preliminary studies were done to standardize and compare the MTT assay with [^{3}H]-thymidine incorporation for fetal rat osteoblasts and rabbit articular chondrocytes. Both assays were linear and reflected numbers of proliferating cells per well 72 hr after initiation of the experiment.

Collagenous and Noncollagenous Protein (NCP) Synthesis. To determine the influence of osteogenin on newly synthesized collagen and NCP synthesis, cultures were labeled with *L*-[2,3,4,5-^{3}H]proline (25 μCi/ml; 4018.2 GBq/mmol; NEN) for the last 6 hr of the culture period as described (21). Cells were then rinsed with isotonic buffer and extracted with 0.5% Triton X-100. After sonication of the sample, protein was precipitated with 10% trichloroacetic acid, washed with acetone, resolubilized in 0.5 M acetic acid, and neutralized with NaOH. An aliquot of cell extract was incubated with 50 units of protease-free bacterial collagenase form III (lot 543-P, Advanced Biofactures, Lynbrook, NY). The labeled proline incorporated into collagenase-digestible protein (CDP) and NCP were determined as described (22). Percent collagen synthesis was calculated after multiplying NCP by 5.4 to correct for the relative abundance of proline in CDP and NCP.

cAMP Response to Parathyroid Hormone (PTH). The response to PTH was monitored by measuring intracellular levels of cAMP. Confluent cultures were incubated in fresh serum-free medium containing 0.2% BSA and a final concentration of 5 × 10^{-4} M isobutylmethylxanthine alone or in combination with bovine PTH (80 ng/ml) for 10 min at 37°C. The reactions were stopped by washing with ice-cold isotonic phosphate-buffered saline, and the cells were extracted with ice-cold propanol (90%) for 1 hr at 4°C. The solution was evaporated in a SpeedVac concentrator (Savant) and frozen at −70°C. Prior to analysis, samples were resuspended and assayed for cAMP content by a radioimmunoassay (Amersham).

[^{35}S]Sulfate Incorporation into Proteoglycans. Cells were labeled with $Na_2{}^{35}SO_4$ (25 μCi/ml) (carrier free, Amersham) for the last 6 hr in culture. The labeled proteoglycans were extracted in 4 M guanidine hydrochloride with 2% Triton X-100 and the medium was analyzed as described (23). Radiolabeled macromolecules were separated from unincorporated radiolabel by gel filtration (23).

Alkaline Phosphatase Assay, Autoradiography of [^{3}H]Thymidine and Cytochemical Staining for Alkaline Phosphatase. Alkaline phosphatase activity was determined (2) in scraped and sonicated cell homogenates at 37°C as described (24). In addition, some cultured cells were fixed in 95% ethanol and stained for alkaline phosphatase and counterstained with Mayer's hematoxylin, as described by the supplier (Sigma, procedure 85).

For bone marrow stromal cell cultures the total number of colonies formed and the number positive for alkaline phosphatase activity were counted in each flask by using a grid. Colonies were grouped according to size into those between 1 and 4 mm in diameter and those >4 mm in diameter at day 16.

In periosteal cells the influence of osteogenin on DNA synthesis in alkaline phosphatase-positive cells was deter-

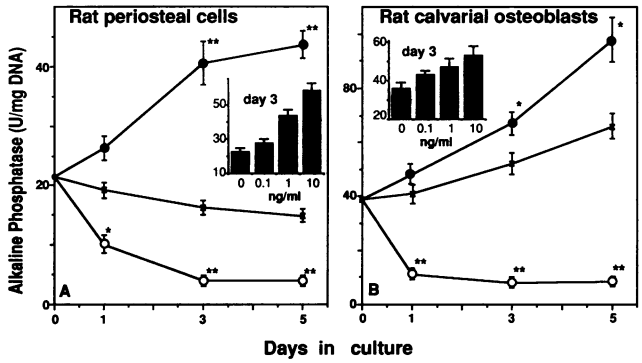

FIG. 1. Alkaline phosphatase activity in periosteal cells (*A*) and calvarial osteoblasts (*B*) treated with osteogenin at ≈2.5 ng/ml (●) or TGF-β_1 at 2.5 ng/ml (○) for 5 days. Controls are indicated by crosses. Dose–response data with three doses of osteogenin (ng/ml) are depicted (*Insets*). Data are mean ± SD of 12 observations from three experiments. U, units. $*P < 0.05$ vs. control. $**P < 0.01$ vs. control.

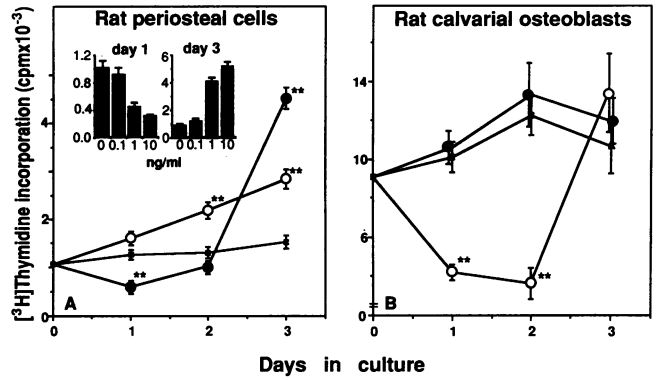

FIG. 2. Time course of the influence of osteogenin at ≈2.5 ng/ml (●) or TGF-β_1 at 2.5 ng/ml (○) on [^{3}H]thymidine incorporation into DNA. Periosteal cells (*A*) and osteoblasts (*B*) were cultured for 3 days in osteogenin and labeled with [^{3}H]thymidine for the last 4 hr of the incubation. Dose–response data with three doses of osteogenin (ng/ml) on days 1 and 3 in periosteal cells are indicated (*Insets*). Data are mean ± SD for eight observations. $**P < 0.01$ vs. control (crosses).

mined 16 and 48 hr after addition of osteogenin. Cells were grown in chambers on slides, and medium was changed to medium containing [^{3}H]thymidine (1.6 μCi/ml). After 16 hr, cells were stained for alkaline phosphatase activity. Cells were then treated twice with 10% trichloroacetic acid for 5 min at 4°C, washed with 100% ethanol, dried overnight in the cold, and exposed to NTB2 nuclear track emulsion (Kodak) at 4°C in the dark for 10 days (25); autoradiograms were developed by standard techniques.

RESULTS

Influence of Osteogenin on Periosteal Cells and Osteoblasts. Treatment with osteogenin resulted in a dose- and time-dependent increase of alkaline phosphatase activity in periosteal cells (Fig. 1). The increase was smaller in osteoblastic cells (Fig. 1). Osteogenin treatment induced the cAMP response to PTH in periosteal cells and a significant stimulation of the PTH responsiveness was observed in osteoblastic cultures (Table 1). Treatment of periosteal cells for 24 hr caused a dose-dependent inhibition of [^{3}H]thymidine incorporation into acid-precipitable DNA, while a continuous exposure for 72 hr resulted in an increase in DNA labeling (Fig. 2). No significant effect on DNA synthesis in osteoblasts was found (Fig. 2). At 2.5 ng/ml, osteogenin increased newly synthesized CDP by 120% and caused a modest increase of NCP in osteoblasts. Collagen synthesis in periosteal cells was increased by 36% but no influence was observed on the synthesis of NCP (Table 2). In periosteal cell populations autoradiograms showed [^{3}H]thymidine incorporation was predominantly located over nuclei of alkaline phosphatase-negative cells 16 hr after the addition of osteogenin, while at 48 hr the grains were located over alkaline phosphatase-positive cells (Table 3). Treatment of both periosteal cell populations and osteoblasts with TGF-β_1 resulted in a dose- and time-dependent decrease in alkaline phosphatase activity (Fig. 1). An inhibition of [^{3}H]thymidine incorporation into acid-precipitable DNA of osteoblasts and stimulation of proliferation in periosteal cells at 48 hr and 72 hr was observed (Fig. 2). TGF-β-neutralizing antibody (40 μg/ml) blocked ≈60% of the activity elicited by TGF-β_1 (1 ng/ml) on the induction of alkaline phosphatase expression in periosteal cells. The same antibody had no effect on the action of osteogenin.

Lack of the Effect of Osteogenin on NIH 3T3 Fibroblasts. Osteogenin did not influence DNA synthesis, alkaline phosphatase activity, or protein synthesis in NIH 3T3 fibroblasts (data not shown).

Stimulation of Proteoglycan Synthesis in Fetal Rat Chondroblasts and Rabbit Articular Chondrocytes. Osteogenin and TGF-β_1 stimulated growth of rat chondroblasts and rabbit articular chondrocytes in micromass cultures (Fig. 3 *A* and *B*). These effects were dose-dependent (data not shown). Treatment with osteogenin resulted in a dose- and time-dependent increase in synthesis of ^{35}S-labeled macromolecules (Fig. 3 *C* and *D*). Treatment with osteogenin (2.5 ng/ml) for 72 hr increased the ^{35}S incorporation into proteoglycans in fetal rat chondroblasts 35-fold and in rabbit articular chondrocytes 11-fold (Fig. 3). In parallel experiments, TGF-β_1 treatment of either cell population initially stimulated the ^{35}S incorporation followed by a dramatic decrease in production of proteoglycans at later time points. Further, the shape of chondrocytes changed to a fibroblastic morphology

Table 1. Influence of osteogenin on PTH-responsive stimulation of cAMP production

Cells	Osteogenin addition	cAMP, pmol per μg of DNA per 10 min
Periosteal cells	−	ND
	+	13.5 ± 1.5
Calvarial osteoblasts	−	22.5 ± 1.5
	+	38.5 ± 3.0*

Cells were plated at 20,000 cells per well. At confluence medium was changed to 0.5% FBS, 0.1% BSA, and 0.2% ITS+ alone (−) or containing osteogenin (2.5 ng/ml) (+). After 72 hr, cAMP was determined 10 min after the addition of PTH. Results are mean ± SD of quadruplicate determinations from three experiments. ND, undetectable.
$*P < 0.01$ vs. control.

Table 2. Influence of osteogenin on CDP and NCP synthesis

Cells	Osteogenin addition	CDP, cpm × 10^{-3}	NCP, cpm × 10^{-3}	% collagen
Periosteal cells	−	7.3 ± 0.7	13.3 ± 1.3	9.2 ± 0.8
	+	9.8 ± 0.2*	14.6 ± 1.8	11.1 ± 0.6
Calvarial osteoblasts	−	5.2 ± 0.1	8.6 ± 0.7	10.1 ± 0.7
	+	12.0 ± 0.2†	13.1 ± 1.8*	14.5 ± 0.6*

Cells were grown to confluence, at which time the medium was replaced with medium containing 0.5% FBS, 0.1% BSA, and 0.2% ITS+ alone (−) or containing osteogenin (2.5 ng/ml) (+). After 42 hr, [^{3}H]proline was added to the cultures for the final 6 hr. Protein synthesis was measured, and results are mean ± SD of 12 observations from three experiments.
$*P < 0.05$ vs. control. $†P < 0.01$ vs. control.

Table 3. Influence of osteogenin on [^{3}H]thymidine incorporation in alkaline phosphatase-positive and -negative periosteal cells

	AP-positive cells, %		[^{3}H]Thymidine-labeled cells, %			
			AP-positive cells		AP-negative cells	
Culture	16 hr	48 hr	16 hr	48 hr	16 hr	48 hr
Control	43 ± 4	38 ± 3	23 ± 3	27 ± 3	28 ± 4	31 ± 5
Osteogenin	45 ± 6	72 ± 6*	28 ± 4	65 ± 6*	58 ± 6*	26 ± 3

Five hundred cells from four coverslip chambers in each group were prepared and then scored for alkaline phosphatase (AP) staining and for nuclear uptake of [^{3}H]thymidine 16 and 48 hr after addition of osteogenin (2.5 ng/ml). Results are a percentage of the total cells scored. The labeled cell was defined as a cell having at least six autoradiographic grains over its nucleus (26).
*$P < 0.01$ vs. control.

confirming the inhibition of expression of chondrogenic phenotype in chondroblasts (27). TGF-β antibody at 40 μg/ml neutralized ≈45% of the TGF-β_1 (1 ng/ml) activity in rat chondroblasts, as measured by proteoglycan synthesis 48 hr after incubation, and had no effect on the activity of osteogenin (1 ng/ml).

Stimulation of Alkaline Phosphatase Activity in Rat Bone Marrow Stromal Cells. The colonies formed by bone marrow stromal cells from rat varied in size and morphology, indicating their heterogenous origin (14). The growth of bone marrow stromal colonies as indicated by MTT assay 72 hr after incubation in the presence of osteogenin was not affected (data not shown). Osteogenin increased the number of alkaline phosphatase-positive colonies; on the other hand TGF-β_1 reduced the number of colonies with alkaline phosphatase activity to low levels and increased the diameter of average colony (Table 4).

DISCUSSION

The foregoing results demonstrate that highly purified osteogenin profoundly stimulates the expression of chondrogenic and osteogenic phenotypes *in vitro*. The results were reproducible with different lots of osteogenin. Osteogenin stimulates alkaline phosphatase activity, a marker of bone cells, in periosteal cells and in bone marrow stromal colonies. The periosteum is an osteoprogenitor cell-containing envelope and may be activated by trauma, certain retroviruses, and tumors to produce cartilage and bone (28). These osteoprogenitor cells are ill defined at present and due to lack of specific markers impossible to distinguish from fibroblastic cells (29). Exposure of periosteal cells to osteogenin for 16 hr resulted in [^{3}H]thymidine incorporation as assessed by autoradiography in alkaline phosphatase-negative cells; by 48 hr alkaline phosphatase was positive in these cells indicating cell differentiation. However, we cannot exclude the possibility that osteogenin stimulates the proliferation and expansion of a select subpopulation of alkaline phosphatase-positive cells.

The clonal origin of colonies of bone marrow stromal cells has been amply demonstrated (15, 16). Whereas osteogenin stimulates the percentage of alkaline phosphatase-positive

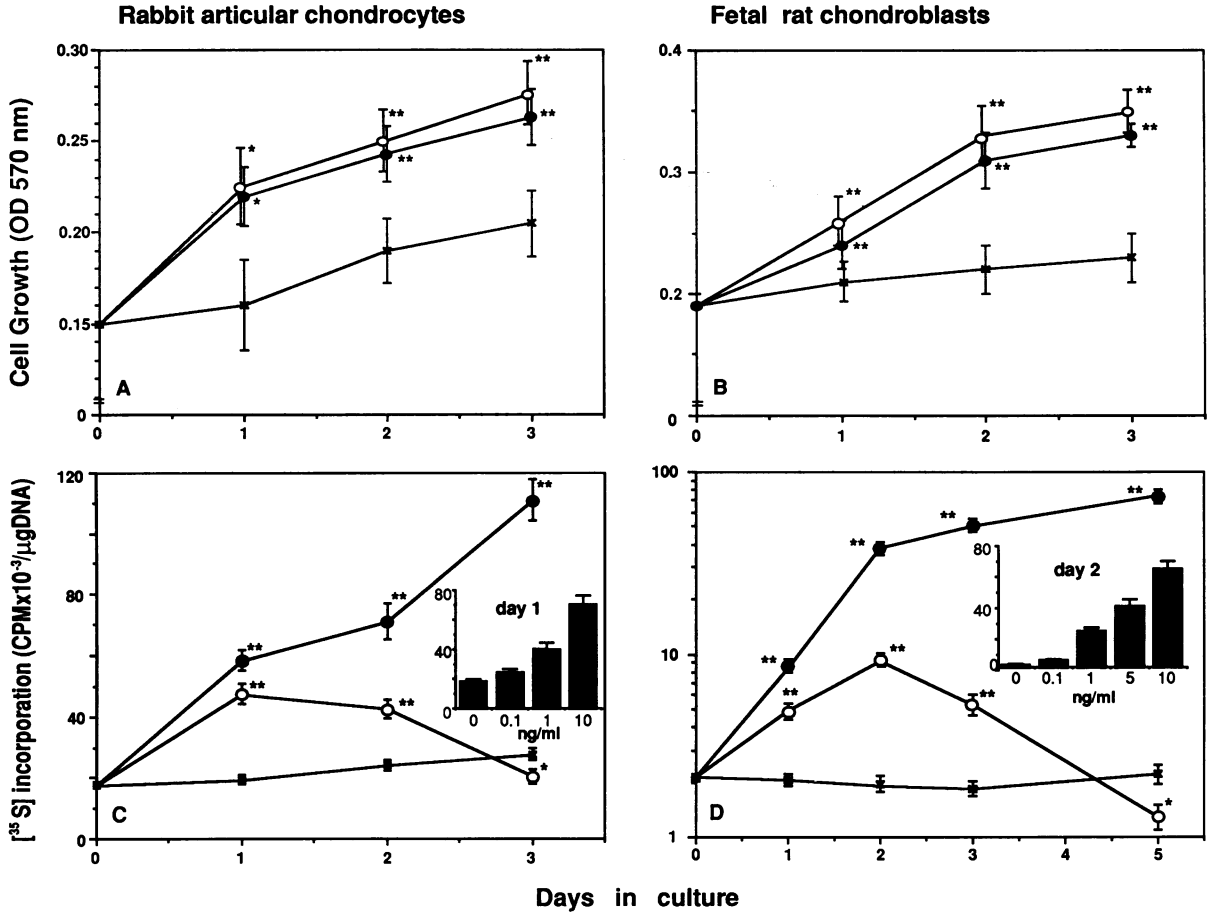

FIG. 3. Influence of osteogenin at ≈2.5 ng/ml (●) and TGF-β_1 at 2.5 ng/ml (○) on cell growth (*A* and *B*) and sulfated proteoglycan synthesis (*C* and *D*) in rabbit articular chondrocytes (*A* and *C*) and fetal rat chondroblasts (*B* and *D*). Cell culture (micromass), MTT colorimetric assay for cell growth, and ^{35}S incorporation were performed. Dose–response data were performed with three or four concentrations of osteogenin and are shown (*C* and *D Insets*). Data are mean ± SD of eight observations. *$P < 0.05$ vs. control. **$P < 0.01$ vs. control (crosses).

Table 4. Influence of osteogenin on alkaline phosphatase expression in rat bone marrow stromal colonies

Group	Size, mm	Colonies, no. per flask	% AP-positive colonies
Control	1–4	115 ± 11	23 ± 3
	>4	37 ± 4	73 ± 8
Osteogenin	1–4	122 ± 12	60 ± 5†
	>4	41 ± 4	77 ± 7
TGF-β_1	1–4	90 ± 8*	12 ± 3†
	>4	68 ± 8†	25 ± 3†

Data are mean ± SD from 12 flasks. Control cultures and cultures treated with osteogenin (2.5 ng/ml) and TGF-β (2.5 ng/ml) for 96 hr between days 14 and 18 after plating the cells were used. AP, alkaline phosphatase.
*$P < 0.05$ vs. control. †$P < 0.01$ vs. control.

clones in bone marrow stromal cells, TGF-β type 1 is inhibitory.

Periosteum-free calvarial cell populations contain various stages of differentiation: preosteoblasts (29, 30), lining cells (31), osteoblasts (32), and osteocytes (33). Increased alkaline phosphatase activity, PTH-stimulated cAMP production, and production of type I collagen are features characteristic of the osteoblastic phenotype in bone and osteosarcoma-derived cells (32, 34). Cells expressing these characteristics are capable of synthesis and assembly of mineralized matrix *in vitro* (35–38) or *in vivo* (39). Osteogenin significantly enhanced the expression of the osteoblastic markers *in vitro* in primary osteoblast cultures. It is noteworthy that there was no influence on the cell proliferation.

Osteogenin increased the biosynthesis of sulfated proteoglycans in fetal rat chondroblasts and in rabbit articular chondrocytes. This stimulatory effect was more pronounced in micromass cultures compared to monolayers (unpublished results). These observations suggest a role for osteogenin in the stimulation and perhaps maintenance of chondrocytic phenotype.

The profound influence of osteogenin in the induction of cartilage and bone in nonskeletal ectopic sites *in vivo* has been demonstrated (1–6), establishing the role of osteogenin in the initiation of differentiation of cartilage and bone. The present work shows a significant influence of osteogenin on the expression of osteogenic and chondrogenic phenotypes *in vitro*, indicating a further role for osteogenin in the maintenance of the newly differentiated phenotypes. Thus these observations will permit further investigation of the mechanism of action of osteogenin and help design pharmaceutical approaches for the initiation of cartilage and bone repair and maintenance of skeletal integrity and homeostasis.

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