Fetal Bovine Bone Cells Synthesize Bone-specific Matrix Proteins

S. WILLIAM WHITSON, WILBUR HARRISON, MARY K. DUNLAP, DANIEL E. BOWERS, JR., LARRY W. FISHER,* PAMELA GEHRON ROBEY,‡ and JOHN D. TERMINE‡

*Department of Biomedical Science, School of Dental Medicine, Southern Illinois University, Alton, Illinois 62002; *Shriner's Hospital for Crippled Children, St. Louis, Missouri 63131; and ‡Mineralized Tissue Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205*

ABSTRACT We isolated cells from both calvaria and the outer cortices of long bones from 3- to 5-mo bovine fetuses. The cells were identified as functional osteoblasts by indirect immunofluorescence using antibodies against three bone-specific, noncollagenous matrix proteins (osteonectin, the bone proteoglycan, and the bone sialoprotein) and against type I collagen. In separate experiments, confluent cultures of the cells were radiolabeled and shown to synthesize and secrete osteonectin, the bone proteoglycan and the bone sialoprotein by imunoprecipitation and fluorography of SDS polyacrylamide gels. Analysis of the radiolabeled collagens synthesized by the cultures showed that they produced predominantly (~94%) type I collagen, with small amounts of types III and V collagens. In agreement with previous investigators who have employed the rodent bone cell system, we confirmed in bovine bone cells that (*a*) there was a typical cyclic AMP response to parathyroid hormone, (*b*) freshly isolated cells possessed high levels of alkaline phosphatase, which diminished during culture but returned to normal levels in mineralizing cultures, and (*c*) cells grown in the presence of ascorbic acid and β-glycerophosphate rapidly produced and mineralized an extracellular matrix containing largely type I collagen. These results show that antibodies directed against bone-specific, noncollagenous proteins can be used to clearly identify bone cells in vitro.

Several criteria have been used to characterize cells isolated and cultured from bone as osteoblasts, although none have proved specific. These are that (*a*) freshly isolated bone cells possess high alkaline phosphatase activity (1), (*b*) bone cells show a strong cyclic AMP response to parathyroid hormone (2), (*c*) bone cells secrete predominantly type I collagen when grown in the presence of ascorbic acid (3), and (*d*) bone cells produce and partially mineralize a matrix, given extended time in culture and defined culture conditions (4–9).

Recently, the isolation and purification of several noncollagenous matrix proteins from fetal calf bone (10) have provided new tools to identify bone cells with increased certainty. Antibodies against these proteins have been used to establish their tissue specificity in fetal calf bone (11–13). In this study we confirm the criteria listed above using bone cells obtained from the calvaria and long bones of fetal calves, the first nonrodent system to be explored. We show by indirect immunofluorescence and in biosynthetic experiments, that these cells produced three bone-specific, noncollagenous proteins; osteonectin (11), the bone proteoglycan (12), and the bone sialoprotein (13), thus proving conclusively that this methodology can clearly identify bone cells in vitro.

MATERIALS AND METHODS

Culture Conditions: Fetal calves (3–5 mo *in utero* [8] and still in the fetal sac) were obtained from Schneider Packing Co., St. Louis, MO within 15 min of being killed. The fetus was removed with stertile gloves and placed in a plastic bag on ice for transport to the laboratory (45 min).

Dissection of the bones was performed under a laminar flow hood. Both halves of the cranial vault, tibias, and femurs were removed under aseptic conditions. The periostea of all bones, the cartilaginous ends of the long bones and the dense connective tissue of the sutures of the calvaria were removed. Radial slices of the cranial vault, 1–2 mm wide and 10–25 mm in length, were removed from the thinnest areas (1–3 mm thick) with a sterilized single-edged razor blade. Longitudinal slices of the tibias and femurs were shaved off the

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 AUGUST 1984 607-614

outer metaphyseal and diaphyseal cortex, rinsed in culture medium without serum, and cut into pieces with approximate dimensions of 1 × 1 × 2 mm. The bone pieces were transferred to 100 mm petri dishes and incubated with intermittent agitation for 40 min at 37°C in 8–10 ml of culture medium without serum and containing 1 mg/ml type IV collagenase (Sigma Chemical Co., St. Louis, MO) or crude collagenase (Worthington Biochemical Corp., Freehold, NJ). The petri dishes were agitated vigorously upon removal from the incubator and the medium containing the released cells placed in 20-ml sterile centrifuge tubes. An equal volume of complete culture medium (see below) was added to the centrifuge tubes to stop the collagenase activity and the tubes centrifuged at low speed for 3–5 min. The medium was removed and the cell pellet resuspended in 2–3 ml of complete medium. ½ ml of the cell suspension (~1 × 10^6 cells) was plated out into 35-mm petri dishes, onto 20-mm^2 glass coverslips or into 75-ml culture flasks and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2.

Each 100 ml of complete media contained 40% Dulbecco's modified Eagle's nutrient media, with 4.5 g/l of glucose, 40% Ham's F-12, 20% heat-inactivated fetal calf serum, 0.1 ml of ITS (500 μg insulin, 500 μg transferrin, 500 μg selenium, Collaborative Research Inc., Lexington, MA), 100 units penicillin and 100 μg streptomycin.

Cells grown to confluency in 75-ml flasks were subcultured by replacing the growth media with Hank's balanced salt solution, pH 7.2 (without calcium and magnesium, and containing 0.2% EDTA). After 5–10 min of incubation at 37°C, the cell suspension was removed, centrifuged, resuspended in 3–5 ml of culture medium and plated out onto six to ten 35-mm petri dishes (~1 × 10^4 cells/dish as determined using trypan blue and a hematocytometer).

To induce mineralization, we stimulated 7–10 d, confluent primary cultures by the addition of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate daily (9). Areas in confluent cultures where most cell overgrowth and cell multilayering occurred mineralized first. Visual observation of the onset of mineralization was possible within 7–10 d and most cultures appeared to add mineralized matrix progressively up to 14 d from the onset. The calcium and phosphorus concentrations of the medium are ~1.73 mM and 1.1 mM, respectively. Mineralized areas were removed manually from the culture dishes and freeze-dried for x-ray diffraction and infrared spectroscopic examination (16).

Alkaline Phosphatase: Samples analyzed were (*a*) freshly-isolated cells and (*b*) cultured cells at confluence treated either with or without β-glycerophosphate and ascorbic acid for 2 to 6 d. The cells were rinsed in Hank's balanced salt solution, scraped off the dish and transferred to test tubes in 2 ml of 0.25 M sucrose and sonicated (Biosonik III [Bronwill Scientific, Rochester, NY] on ice for 30 s at the high setting.

Alkaline phosphatase activity of the sonicated solution was determined using the Sigma procedure and reagents (Sigma Chemical Co.) #246-A. *P*-Nitrophenyl phosphate (66 μM) was added to 6.5 ml of a 0.1 M solution of the buffer 2-amino, 2-methyl, 1-propanol at pH 10.2 which contained (0.1 mM) magnesium. The alkaline phosphatase activity was determined by the difference in the 405-nm absorbance between 0 and 3 min of incubation at 37°C.

Cyclic-AMP Response to Parathyroid Hormone: At culture confluency, the medium was drawn off 35 mm dish cultures and replaced by phosphate-buffered saline containing 5 × 10^{-4} M 3-isobutylmethyl xanthine, a phosphodiesterase inhibitor that prevents the enzymatic breakdown of cyclic AMP. After 10–15 min of incubation, the buffer was replaced by culture medium containing 0.5% serum albumin, 3-isobutylmethyl xanthine and 100 ng/ml parathyroid hormone, 1-34 fragment (Beckman Instruments, Inc., Palo Alto, CA). Groups of cultures were incubated for 1, 2.5, 5, 10, and 20 min at 37°C. Cyclic AMP production was terminated by pouring off the medium, rinsing twice with phosphate-buffered saline, adding ice-cold trichloroacetic acid (10%) to the culture and placing the dish on ice for 30 min. The trichloroacetic acid precipitate was scraped from the dishes, and with the trichloroacetic acid transferred to centrifuge tubes, sonicated, and spun down. The supernatant, containing the cyclic AMP, was washed five times with equal volumes of diethyl ether to remove trichloroacetic acid and lyophilized. The amount of cyclic AMP present was determined by the protein binding method of Gilman (17) as previously modified (12). The results are expressed in picomoles cAMP/culture.

Immunofluorescence Microscopy: Immunofluorescence staining was carried out on bone cells grown on glass coverslips. Staining for osteonectin, bone sialoprotein, bone proteoglycan and type I collagen was done on 3–5-d nonconfluent primary and passaged cultures, confluent cultures grown in complete medium, or confluent cultures to which ascorbic acid and β-glycerophosphate were added daily for periods up to 4 d. The coverslips were removed from the culture media, rinsed three times in 50 mM phosphate-buffered saline and fixed for 1 min in absolute methanol and air-dried. Coverslips containing mineralized matrix were demineralized first for 24 h with 0.1 M EDTA as described earlier (11) before staining.

The coverslips were incubated in a moist chamber with a 1:10 dilution of antibodies to type I collagen (a gift from Dr. G. R. Martin, National Institute of Dental Health, National Institutes of Health), osteonectin (11), bone proteoglycan (12), bone sialoprotein (13) and preimmune rabbit serum (control) for 1 h at room temperature. After incubation, the coverslips were washed in four changes (5 min each) of phosphate-buffered saline with gentle agitation for a total of 20 min. The coverslips were briefly immersed in deionized-distilled water to prevent any precipitation of salts and allowed to air dry. The staining and washing procedures were repeated with a 1:20 dilution of fluorescein-conjugated IgG, Fc fragment (anti-goat made in sheep for type I collagen and anti-rabbit made in goat for osteonectin, bone proteoglycan, and bone sialoprotein [Cappel Laboratories, Cochranville, PA]). Coverslips were mounted on glass slides in 85% glycerol and examined with a Leitz-Ortholux II photomicroscope with a fluorescence vertical illuminator. Photomicrographs of antibody-treated and controls were exposed and processed under identical conditions.

Noncollagenous Protein Biosynthesis: Primary cultures were passed into 35-mm petri dishes and grown to confluency. The medium were removed and the cultures washed with Dulbecco-Vogt medium with 4.5 g of glucose/liter without methionine or fetal calf serum. For osteonectin, 10 μCi/ml of L-[^{35}S]methionine, (800 Ci/nmol, translation grade [New England Nuclear, Boston, MA]) was added to one ml of the Dulbecco-Vogt medium and incubated for 4 h at 37°C. For bone sialoprotein, to 1 ml of Dulbecco-Vogt plus 20% fetal calf serum, 10 μCi/ml of L-[^{35}S]methionine was added and incubated at 37°C for 24 h. For bone proteoglycan, 100 μCi/ml of $^{35}SNa_2SO_4$ (carrier free [ICN Pharmaceuticals, Inc., Cleveland, OH]) was added to 1 ml of the Dulbecco-Vogt and incubated for 4 h at 37°C. After incubation with label, the medium was removed and an 800-μl aliquot was incubated with 100 μl Pansorbin TM (*Staphylcoccus aureus* cells) (Calbiochem-Behring Corp., San Diego, CA) that had been pre-washed with buffer A (10 mM Tris, 0.9% NaCl, pH 7.4, 0.5% Nonidet P-40) for 1 h on ice. The solution was centrifuged and the pellet discarded. The appropriate antisera (10 μl) was added to the supernatant fraction and incubated on ice for 1 h. An additional 100 μl of fresh, washed Pansorbin was added and incubated on ice for 1 h. The solution was centrifuged, and the supernatant discarded. The pellet was washed three times by resuspending in buffer B (buffer A plus 18.44 g KCl/100 ml) and centrifuged after each wash. The pellet was washed twice further with buffer A and a final time with distilled H_2O. The pellet was resuspended in 50 μl of gel sample buffer as described by Laemmli containing β-mercaptoethanol (19), boiled for 3 min and centrifuged. The supernatant fraction was applied to a 4–20% polyacrylamide SDS gel (3% stacking gel) (12) and electrophoresed until the bromophenol blue tracking dye was within 1 cm of the bottom. The gel was equilibrated with Autofluor (National Diagnostics, Inc., Somerville, NJ) according to instructions, dried and a flurogram made using Kodak XAR film at −70°C. After development, the fluorogram was scanned on an LKB Ultrascan, Laser Densitometer, model 2202 (LKB Instruments, Inc., Gaithersburg, MD).

Collagen Biosynthesis: Primary bone cell cultures were derived as above, passed and grown to confluency in complete media in 35-mm petri dishes. Collagen was radiolabeled and purified essentially as described by Rowe and Shapiro (20), but with several modifications. The cultures were provided with fresh Dulbecco's modified Eagle's medium, containing 20% fetal bovine serum and 50 μg/ml of ascorbic acid, 24 h before labeling. The labeling medium (2 ml/plate) contained Dulbecco's modified Eagle's medium without glutamine, with 20% dialyzed fetal bovine serum (Gibco Laboratories, Grand Island, NY), 1 unit of penicillin/ml, 1 μg streptomycin sulfate/ml, 50 μg ascorbic acid/ml, 50 μg 2-aminopropionitrile (Sigma Chemical Co.) and 25 μCi of 3-5-[^{3}H]-proline (Amersham Corp., Arlington Heights, IL)/ml. After 16 h, the medium was drawn off and the cell layer removed by scraping in 1 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.3 mM phenylmethylsulfonyl fluoride, 20 mM EDTA acid, and 10 mM *N*-ethylmaleimide. The medium and cell layer fractions were combined, homogenized and centrifuged at 10,000 *g* to remove insoluble material. Trichloroacetic acid was added to a final concentration of 15% to the supernatant fraction, and the resulting precipitate was washed with 5% trichloroacetic acid and subsequently dissolved in 0.5 M acetic acid. Lathyritic rat skin collagen was added (50 μg/ml) as a carrier before treatment with pepsin (Calbiochem-Behring Corp., 0.1 mg/ml) to digest noncollagenous proteins. After stirring at 4°C for 24 h, collagen was precipitated from the 0.5 M acetic acid solution by the addition of solid NaCl to 1.7 M. The precipitates were collected by centrifugation at 10,000 *g* for 20 min, washed with 4.5 M NaCl, 0.05 M Tris-HCl, pH 7.4, and finally with 18% ethanol. After drying, the precipitates were electrophoresed on SDS 5% polyacrylamide slab gels (19) with and without reduction by 1 mM dithiothreitol and with delayed reduction with dithiothreitol (21) to separate α 1(I) from α 1(III). The gels were prepared for fluorography by using dimethylsulfoxide and diphenyloxazole (22) and were exposed to Kodax XAR film at −70°C for 48 h. Quantitation of the different α-chains was determined by scanning the film with a laser densitometer as described above.

Electron Microscopy: Bone cells were grown either on coverslips or

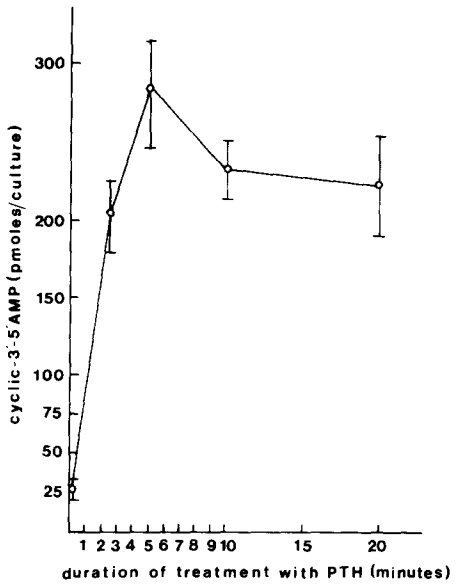


FIGURE 1 Confluent cultures of fetal bovine bone cells elicit a typical cyclic 3',5'-AMP response to 100 ng/ml of parathyroid hormone (1–34 fragment). Peak response is demonstrated at 5 min. Each point represents the mean from four cultures, each analyzed in duplicate.

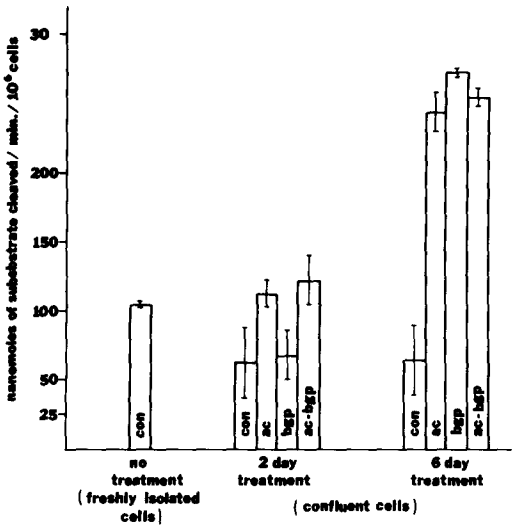


FIGURE 2 Confluent cultures of fetal bovine bone cells demonstrate minimal alkaline phosphatase activity either basally or after stimulation with ascorbic acid (50 μg/ml) and/or β-glycerophosphate (10 mM), both for 2 d. However, after 6 d of treatment cultures each treatment a significant rise in alkaline phosphatase activity, comparable to that measured in freshly isolated cells. Each bar value represents the mean from three cultures each analyzed in duplicate. *con*, control. *ac*, ascorbic acid. *bgp*, β-glycerophosphate.

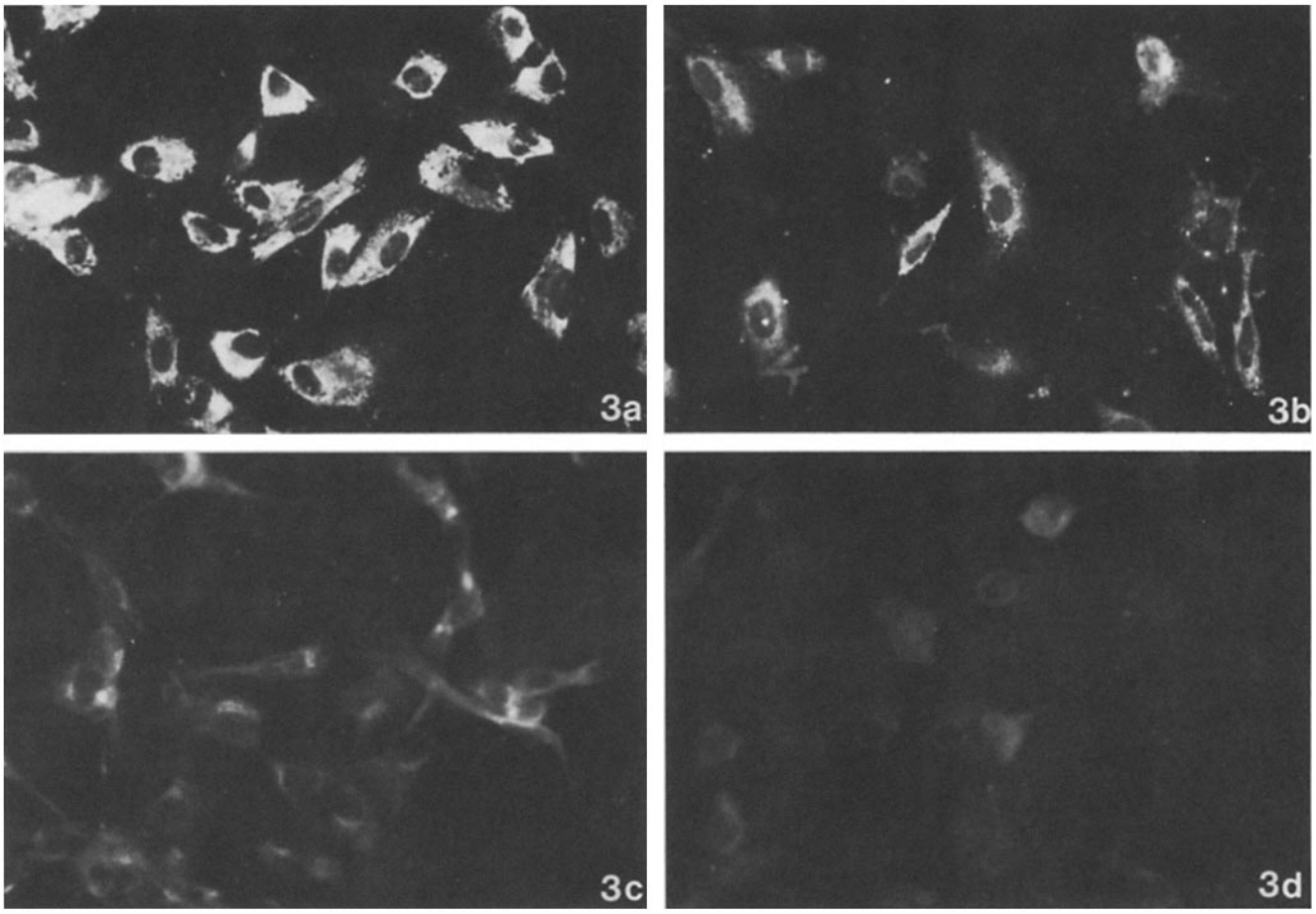
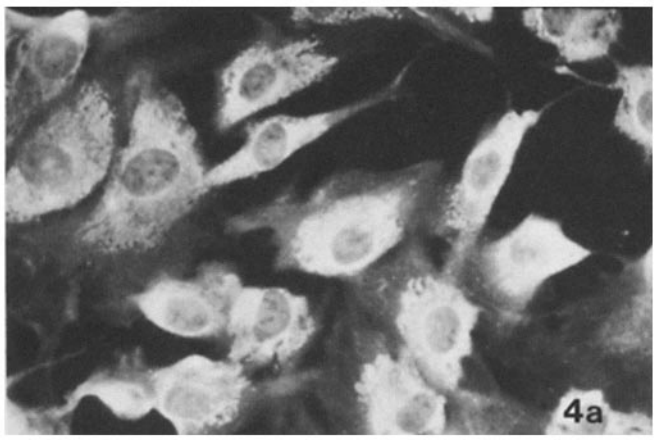
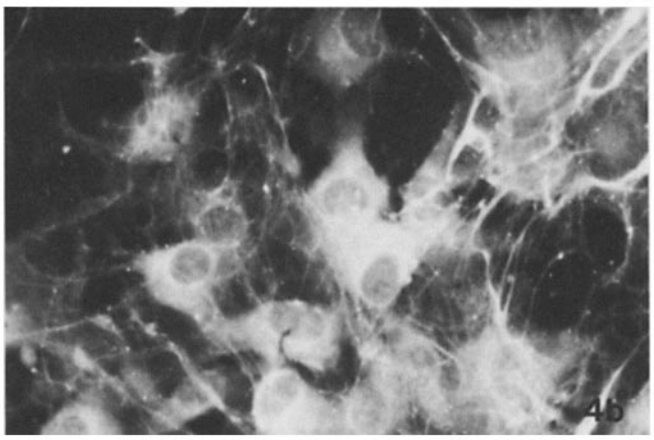


FIGURE 3 Indirect fluorescein immunofluorescent micrographs of 5-d cultures of bone cells exposed to antibodies to bovine osteonectin (*a*), bone proteoglycan (*b*), bone sialoprotein (c), and a representative preimmune serum control (*d*). The osteonectin response is very strong throughout most of the cytoplasm, while both the bone proteoglycan and bone sialoprotein appear to be limited to the Golgi region. × 300.





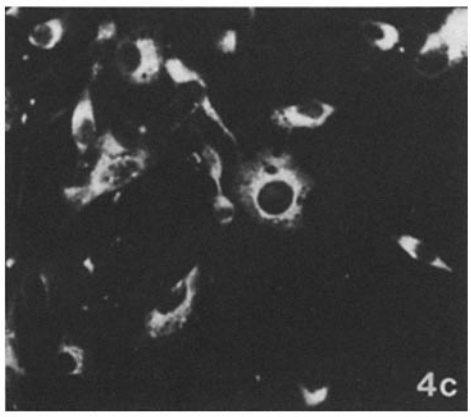


FIGURE 4 Indirect fluorescein immunofluorescence of antibodies directed against type I collagen and osteonectin after the addition of 50 μg/ml ascorbic acid in nonconfluent cultures. In the absence of added antibodies to type I collagen stain the cell cytoplasm, primarily (*a*), while both the cells and the extracellular matrix stain when ascorbic acid is added to the culture medium (*b*). Osteonectin (*c*) remains localized primarily to the perinuclear region with ascorbic acid treatment (compare to Fig. 3*a*).

in petri dishes with flexible plastic inserts (Falcon Labware, Oxnard, CA). Fixation took place in 1.0% glutaraldehyde in 0.1 M phosphate-sucrose buffer, pH 7.3, for a ½ h at room temperature. Postfixation was for 1 h in 1% phosphate-sucrose-buffered osmium tetroxide at 4°C. The cells were dehydrated in an alcohol series and embedded in an epoxy resin. 1–2 μm thick sections were prepared for light microsocpy and stained with toluidine blue and basic fuschin. Thin sections (90–100 nm) were cut with a diamond knife (Dupont Instruments, Inc., Wilmington, DE) on an LKB ultramicrotome. The sections were collected on copper grids and stained with uranyl acetate and lead citrate and viewed at initial magnifications of 3,000–10,000 on a Philips 300 electron microscopy at 60 kV.

Von Kossa Staining: Mineralization in ascorbic acid and β-glycerophosphate-treated cultures was detected by Von Kossa's silver method (23). The cultures were treated with 5% silver nitrate for 30 min, rinsed and counterstained for 60 s in 0.3% toluidine blue.

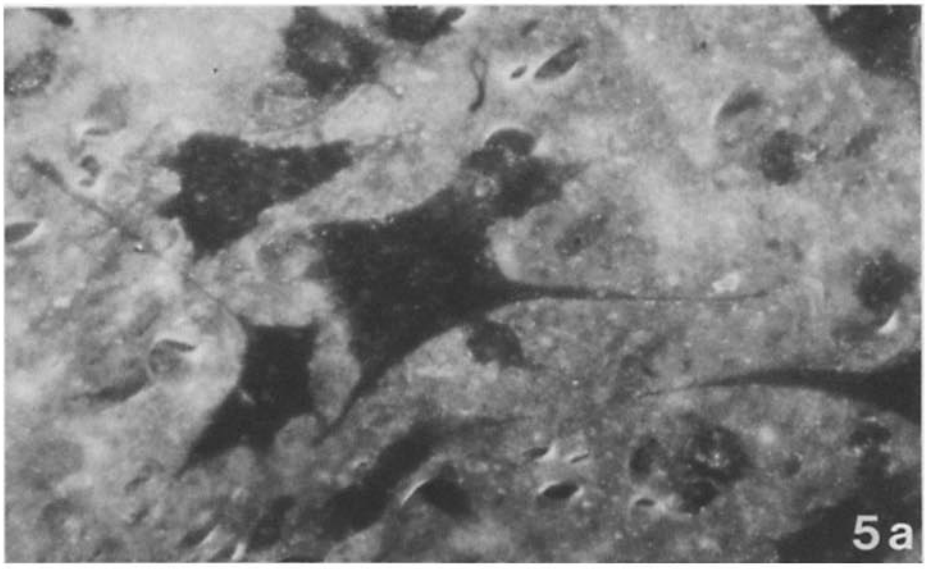
RESULTS

Under the isolation and culture conditions employed, confluent cells obtained from either calvaria or outer cortices of long bones all showed a typical increase in cyclic 3′,5′-AMP in response to parathyroid hormone (Fig. 1). Alkaline phosphatase levels were high at isolation (Fig. 2) but fell after 2 d in culture and remained depressed during subsequent culture. When medium containing either 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, or both was added daily to confluent cultures, alkaline phosphatase levels again became elevated after 6 d of treatment (Fig. 2). The combination of ascorbic acid and β-glycerol phosphate induced the best stimulation; however, stimulation by the two substances simultaneously was not additive (Fig. 2).

5-d primary or secondary cultures of the bovine bone cells were exposed to antibodies specific for the bovine bone proteins: osteonectin; the bone proteoglycan; and the bone sialoprotein. In all cases, the cells were positive for each protein (Fig. 3, *a–c*) when compared to the preimmune serum control (3*d*). Osteonectin immunofluorescence was most intense (Fig. 3*a*), while that of both the bone proteoglycan and the bone sialoprotein was distinct but less intense (Fig. 3, *b* and *c*). These cells were also strongly positive to antibodies against type I collagen (Fig. 4, *a* and *b*). In the absence of ascorbic acid, fluorescence was visible intracellularly (Fig. 4*a*). With the addition of ascorbic acid to the cultures, fluorescence was observed both intracellularly and extracellularly along matrix fibrils (Fig. 4*b*). In contrast, little, if any, extracellular fluorescence was seen when ascorbic acid–treated cultures were exposed to the osteonectin antibody (Fig. 4*c*), presumably because it was released into the media. However, the cytoplasmic distribution of osteonectin staining appeared more perinuclear in the ascorbic acid–treated cells (compare Figs. 4*c* and 3*a*).

Confluent cultures, placed on ascorbic acid and β-glycerophosphate produced and mineralized an extracellular matrix, commencing 7 d after the start of treatment. The matrix

FIGURE 5 Indirect fluorescein immunofluorescent micrographs of demineralized, in vitro bone matrix, treated with antibodies to type I collagen (*a*), osteonectin (*b*) or with preimmune serum (*c*). Because of the extended period of tissue treatment required to demineralize the matrix, the remaining cell remnants present dark (fluorescein negative) profiles. × 300.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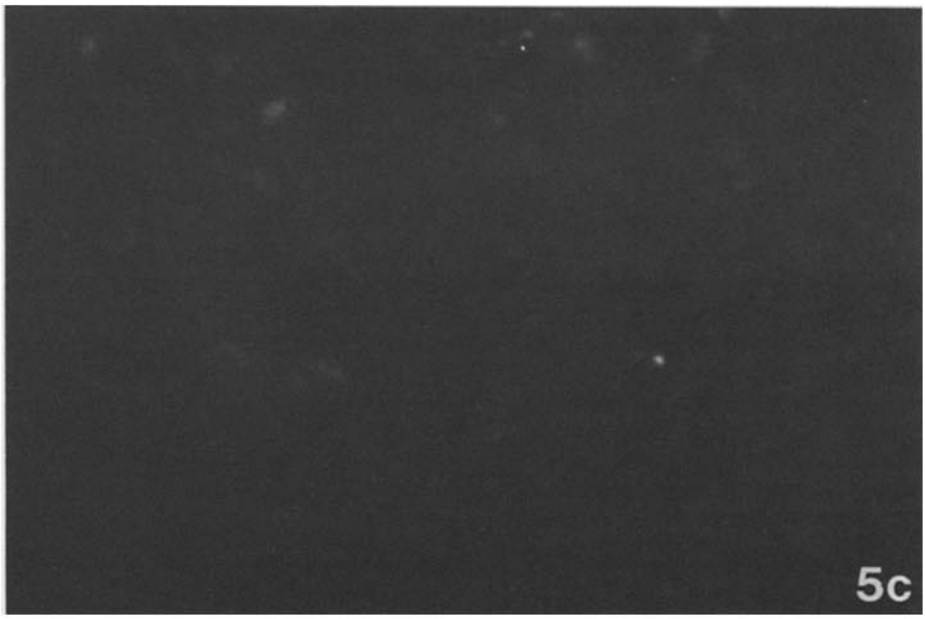
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production and mineralization were supported for 3–4 wk in culture. The mineral present was shown to be hydroxyapaptite by x-ray diffraction and infrared spectroscopy (data not shown). When this in vitro-produced matrix was demineralized and stained immunocytochemically for osteonectin and type I collagen, it was positive for both (Fig. 5). We surmise that anti-osteonectin fluorescence is less intense than collagen because of its loss during demineralization. In addition, during processing cells were lost from the matrix surface and the cells which remained retained little or no fluorescence.

To confirm that the cultured cells produce both noncollagenous and collagenous bone matrix proteins, we labeled cultures with either [^{35}S]methionine, Na$^{35}SO_4$, or [^{3}H]proline. Fluorograms ([^{35}S]methionine) of SDS gels from labeled media immunoprecipitated with anti-osteonectin (Fig. 6*a*) or anti-bone sialoprotein (Fig. 6*b*) indicate synthesis and secretion of these two proteins. Similary, anti-bone proteoglycan immunoprecipitate ($^{35}SO_4$) (Fig. 7) showed the production of this macromolecule by the bovine osteoblast cultures.

Fluorograms of SDS gels of the collagens produced by passaged confluent bovine osteoblasts (Fig. 8) showed that similar amounts and types of collagen were produced by cultures from both calvaria and long bones. Quantitation of types I and III collagens was accomplished by the use of delayed reduction with dithiothreitol to separate α 1(I) from α 1(III) chains. All cultures produced approximately 94% type I and approximately 6% type III collagen. Long-term exposure of the gels to x-ray films revealed that a small amount (0.5%) of type V collagen was also produced as indicated by the presence of α1(V) chains (data not shown).

The ultrastructure of bone cells stimulated with ascorbic acid and β-glycerophosphate exhibited cytoplasmic elements consistent with an active connective tissue secretory cell (Fig. 9). Collagen fibrils were abundant adjacent to the cells.

Mineralization in confluent cultures of cells treated for up to 14 d with β-glycerophosphate and ascorbic acid was confirmed by the Von Kossa method (Fig. 10). Small focal points of mineralization which were visable on day 7, enlarged and fused with one another until about two-thirds of the culture dish was covered with mineralized matrix.

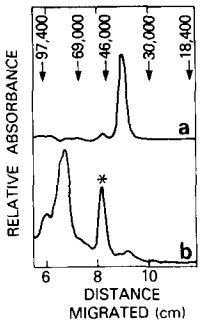

FIGURE 6 Anti-osteonectin (*a*) and anti-bone sialoprotein (*b*) immunoprecipitate fluorograms of SDS gels from medium of cultured osteoblasts labeled with [^{35}S]methionine. Scan (*a*) contains a peak at ~38,000 mol wt, the location of authentic osteonectin on the SDS gradient (4–20%) polyacrylamide gel system. Scan (*b*) shows a broad peak at ~80,000 mol wt, a position identical to that of the fetal bovine bone sialoprotein used as original antigen. The asterisk denotes a band precipitated nonspecifically in all [^{35}S]methionine-labeled preparations by the Pansorbin treatment. ^{14}C-molecular weight markers include phosphorylase B (97,400), bovine serum albumin (29,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lactoglobulin A (18,400).

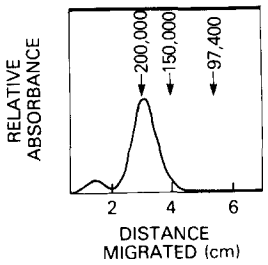

FIGURE 7 Anti-bone proteoglycan immunoprecipitate fluorogram (scan) of an SDS gel from media of cultured osteoblasts labeled with $^{35}SO_4$. Both the 190,000-mol-wt peak (3-cm migration) and the minor peak at 1-cm migration are characteristic of the electrophoretic pattern for purified fetal bovine bone proteoglycans on this SDS gradient gel system (6). ^{14}C-Molecular weight markers include myosin (200,000), γ-globulin (150,000), and phosphorylase B (97,000).

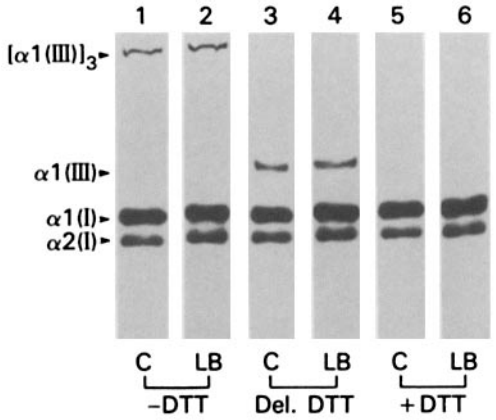

FIGURE 8 Separation of α-chains isolated from bone cell cultures by SDS gels. [^{3}H]Proline-labeled collagens from calvaria (C) and long bone (*LB*) cell cultures were electrophoresed: in the absence of the reducing agent, dithiothreitol (*–DTT*, lanes *1* and *2*); with delayed reduction following 1½ h of electrophoresis (*Del. DTT*, lanes *3* and *4*); and with reduction at the start of electrophoresis (*±DTT*, lanes *5* and *6*). Without reduction α1(I) and α2(I) chains (type I collagen) migrate into the gel while (α1[III])$_3$ chains (type III collagen) remain at the top of the gel (lanes *1* and *2*). With continuous reduction, α1(III) and α1(I) co-migrate (lanes 5 and 6), but are separated from one another upon delayed deduction with dithiothreitol (lanes *3* and *4*).

DISCUSSION

This study showed that bone cells, isolated from fetal calf calvarial and long bones by conventional means, synthesized substantial quantities of three tissue-specific noncollagenous proteins of fetal calf bone; osteonectin, bone proteoglycan and bone sialoprotein. These cells can, therefore, be identified as functional osteoblasts. The bovine bone cells also were positive for alkaline phosphatase upon isolation from the tissue, produced cyclic AMP upon stimulation with parathyroid hormone, synthesized type I collagen, and mineralized their matrix when treated with β-glycerophosphate and ascorbic acid. Thus, all criteria established earlier for presumptive bone cell identity (1–9) were also expressed by the bovine osteoblasts described in this study.

Although the precise function of the three noncollagenous

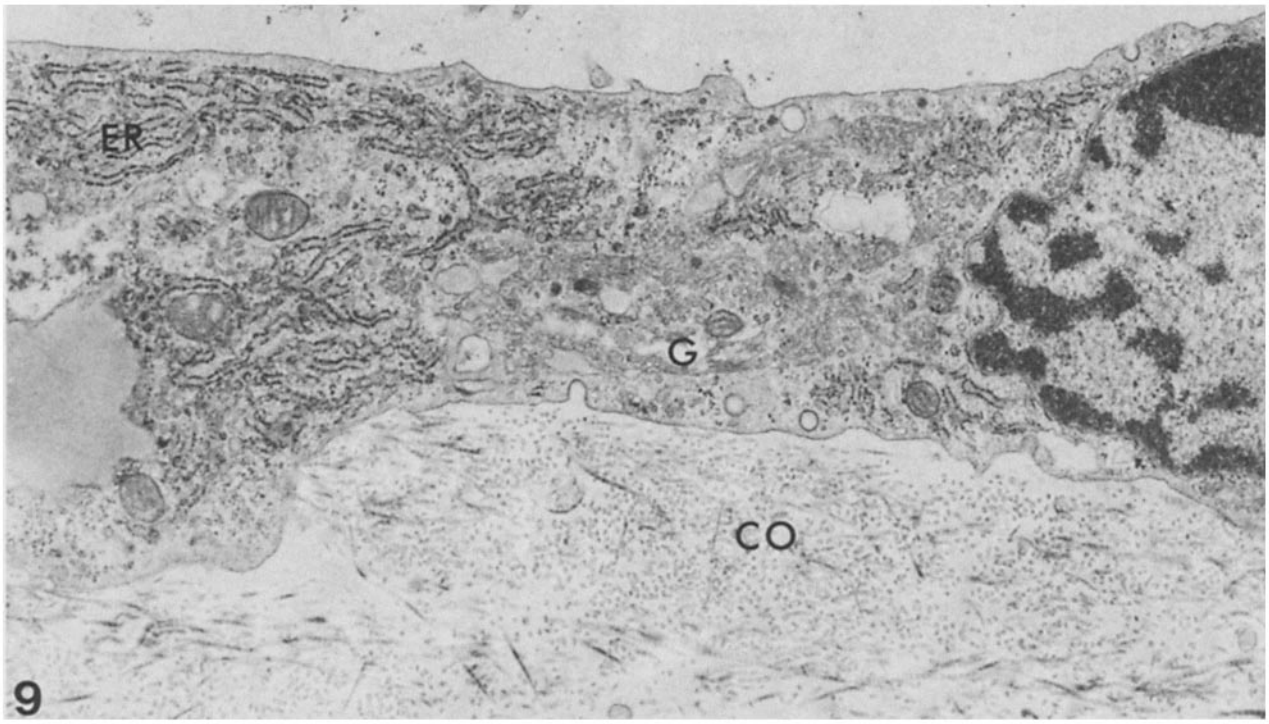

FIGURE 9 Osteoblast from a confluent culture exposed for 6 d to 50 μg/ml ascorbic acid and 10 mM β glycerol phosphate. The cytoplasm of the cell is rich in rough endoplasmic reticulum (*ER*) and contains a prominent Golgi apparatus (*G*). Large numbers of collagen fibrils (*CO*) are found extracellularly. × 33,000.

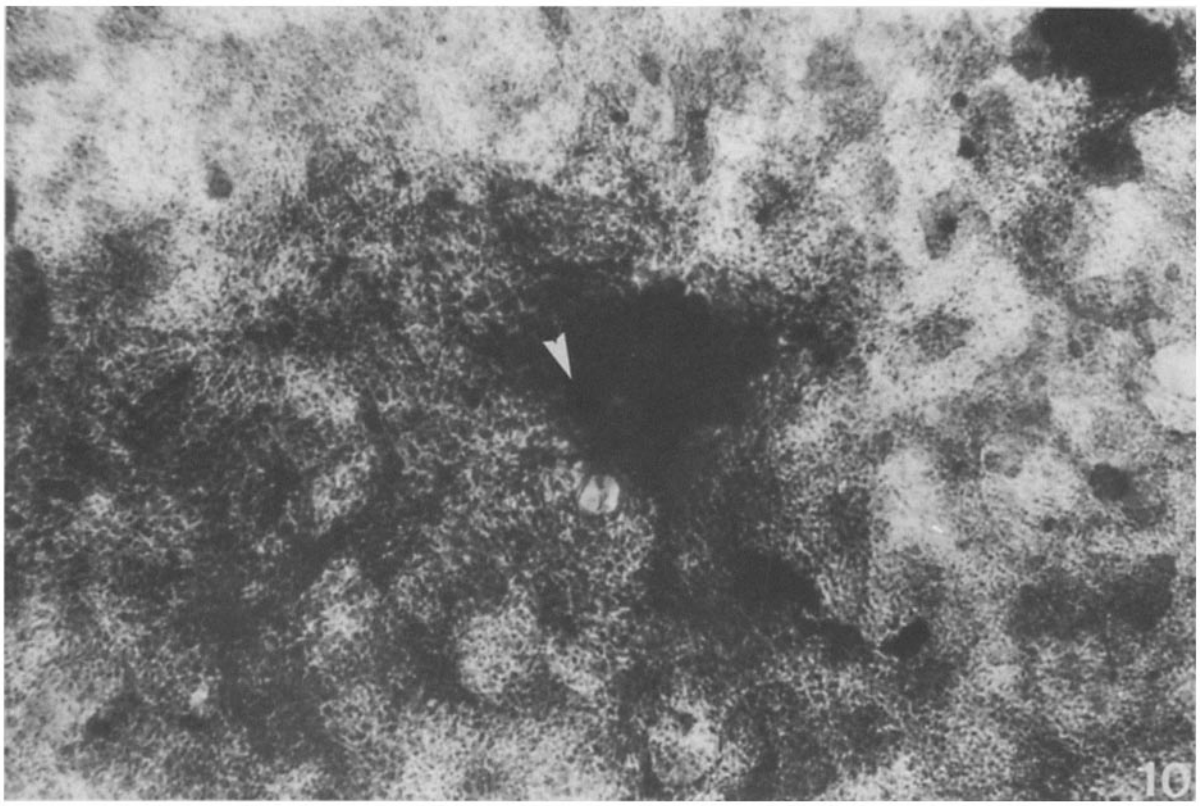

FIGURE 10 Von Kossa/toluidine staining of a confluent culture treated with ascorbic acid and β-glycerophosphate for 10 d. Focal points (arrow) of heavy mineralization are found within areas of more diffuse mineralization. × 128.

bone proteins is unknown, they appear to be necessary for the formation of normal, structurally-sound bone tissue. In one form of bovine osteogenesis imperfecta, now being employed as a model for human osteogenesis imperfecta, osteonectin and the bone proteoglycan are reduced by >90% and the bone sialoprotein by 40–50% (unpublished data). Because of the relatively small size of the bone proteoglycan molecule as compared to the cartilage proteoglycan aggregate (12), it is likely that it does not prevent mineralization but, provides another as yet undiscovered function.

The alkaline phosphatase data shows that either ascorbic acid or β-glycerophosphate can induce alkaline phosphatase activity in confluent primary cultures of bovine bone cells. The mechanism of how either induction occurs must remain speculative. The ascorbic acid mechanism might be related to an increase in membrane turnover (presumably also including alkaline phosphatase) brought about by an increase in matrix synthesis and release. β-Glycerophosphate was originally used to induce mineralization in vitro (9, 15) because it has been used successfully both chemically and histochemically as an alkaline phosphate substrate. Perhaps β-glycerophosphate operates through a substrate induction mechanism. Addition of ascorbic acid to the nutrient medium appeared to be essential for detection of type I collagen and osteonectin (Fig. 4*b*, 4*c*) in the extracellular matrix of confluent cultures.

Our experience supports the findings that significant mineralization in tissue culture occurs only after confluency has been reached and substantial cell overgrowth and multilayering has occurred (7–9). In addition, mineralization appears to require a certain volume of matrix. It has been our consistent observation that before mineralization onset, β-glycerol phosphate and ascorbic acid together produce a much greater matrix volume and in a shorter time period than do either alone. This results in appearance of mineral within 14 d in culture in agreement with Ecarot-Charier et al. (9), 1–2 wk earlier than others have reported (4–8).

As others have shown in rodent cell culture (8), mineralization of bovine bone matrix occurs in matrix sandwiched between two bone cell layers, one a single cell layer which adheres to the dish, the other a continuous multicellular layer that sits atop the matrix. At the initiation of mineralization focal points of mineral appear and expand in size in areas where the cell layer atop the matrix remains as a multicellular layer (unpublished observations).

The small amounts of type III collagen found are as expected, considering that reticular connective tissue associated with numerous blood capillaries is positive for type III collagen in fresh frozen sections of undemineralized fetal calf bone (unpublished findings). In addition, interstitial (progenitor) mesenchyme lying between the forming bone spicules in intramembranous bone formation areas (calvarial, subperiosteal bone) was also positive for type III collagen (12). Bone cell cultures were previously shown (24) to contain small amounts of type V collagen. The absence of type II collagen from the cultures using indirect immunofluorescent microscopy suggests that they were free of cartilage cell contamination.

Experiments designed to show that the bovine bone cells produced bone gla protein (osteocalcin) gave poor results by both indirect immunofluorescent antibody staining and biosynthetic labeling. This failure could, however, be related to the young age of the fetuses from which the cells were isolated. It is known that in the fetal bones of at least three species, osteocalcin synthesis does not reach substantial levels until birth and early neontal life (25–27). It is noted that the only cells unequivocally shown to produce osteocalcin are rat osteosarcoma cell lines of Rodan (28), the precise origin of which remains unknown.

Received for publication 8 August 1983, and in revised form 17 April 1984.

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