Osteoblasts Isolated from Mouse Calvaria Initiate Matrix Mineralization in Culture

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ABSTRACT A method is presented for isolating osteoblasts from newborn mouse calvaria without the use of digestive enzymes. The procedure is based on the ability of osteoblasts to migrate from bone onto small glass fragments (Jones, S. J., and A. Boyde, 1977, *Cell Tissue Res.*, 184:179–193). The isolated cells were cultured for up to 14 d in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μg/ml of ascorbic acid. 7-d cultures were incubated for 24 h with [^{3}H]proline. High levels of collagen synthesis relative to total protein were found, as measured by collagenase digestion of medium and cell layer proteins. Analysis of pepsin-digested proteins from the same cultures by SDS PAGE showed that type I collagen was predominantly produced with small amounts of type III and V (α1 chains) collagens. Osteoblasts grown in the presence of β-glycerophosphate were able to initiate mineral deposition in culture. Electron microscopic analysis of the cultures revealed the presence of needle-shaped apatite-like crystals associated with collagen fibrils and vesicles in the extracellular space. Mouse skin fibroblasts cultured under identical conditions failed to initiate mineralization. Electron histochemical studies revealed the presence of alkaline phosphatase activity, associated with osteoblast membranes, matrix vesicles and on or near collagen fibrils. Thus these isolated osteoblasts retained in culture their unique property of initiating mineralization and therefore represent a model of value for studying the mineralization process in vitro.

Many attempts have been made to isolate osteoblasts that will retain in culture the specific function of bone-forming cells, that is the synthesis of a calcified type I collagen matrix. Such an in vitro system would provide a potent tool for investigating the biochemical aspects of bone formation and the hormonal regulation of this process.

Since the work of Peck et al. (1), many enzymatic procedures (2–8), microdissection (2, 7–9), electrophoretic (10), and cloning (11, 12) techniques have been developed for isolating a cell population that expresses osteoblastic properties. However, most of the culture systems described failed to elaborate a mineralized matrix and provisional identification of the cells as osteoblasts has been based on the study of biochemical parameters believed to be characteristic of osteoblasts in situ, primarily responsiveness to hormones (3–7, 12–14). Mineralization has been reported in a cell culture derived from osteoblasts of rat calvaria (11) but, in the same study, mineral deposition was also observed in rat fibroblasts cultured under similar conditions.

We describe here a method for isolating from newborn mouse calvaria osteoblasts that retain in culture the ability to form a calcified type I collagen matrix. Mineralization occurring in cultures grown in the presence of β-glycerophosphate (15, 16) was studied by electron microscopy. Control mouse fibroblasts did not show mineral deposition.

MATERIALS AND METHODS

Isolation and Culture of Bone Cells: The isolation technique is based on the ability of osteoblasts to migrate from bone onto glass fragments as described by Jones and Boyde (17).

Calvariae (frontal and parietal bones) from 5–6-d-old mice (C57BL/6J strain) were removed aseptically. The periosteal layers on both sides were carefully stripped off with tweezers under Dulbecco's modified Eagle's medium buffered with 15 mM HEPES, pH 7.4 (DME) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). Calvariae were transferred to Lux Scientific P60 Petri dishes (4 calvariae/dish) containing 6 ml of DME supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Rockville, MD) and 50 μg/ml of ascorbic acid. Glass fragments (0.18-mm thick, around 1-mm^2 surface area) obtained from coverslips (Corning Glass Works, Corning, NY) were placed on

the endocranial surface, avoiding suture areas. After 4 d in culture, cells on glass fragments were scraped and grown in the culture medium described, with or without β-glycerophosphate (5 or 10 mM) for up to 14 d. The medium was changed every 2-3 d. Day 1 was taken as the first day of culture after cell removal from the glass fragments.

Fibroblast Cultures: The fibroblast cultures were derived from cells growing out of skin fragments taken directly over the calvaria of a 5-6-d-old mouse. They were maintained in DME supplemented with 10% FCS. Confluent cultures between the second and sixteenth passage were used in these experiments. Primary cultures were also used for mineralization studies.

Collagen Analysis: After culture for various times (1 to 14 d), cells were labeled for 24 h with 50 μCi/ml of 5-[³H]proline (New England Nuclear, Boston, MA; 7.8 Ci/mM) in DME containing 10% FCS and 100 μg/ml each of ascorbic acid and β-aminopropionitrile fumarate. Following labeling, protease inhibitors, at a final concentration of 25 mM EDTA, 10 mM N-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride (PMSF), were added to the culture medium to prevent proteolysis. The medium was then made 0.5 M with respect to acetic acid and the cells dispersed in the medium with a rubber policeman. After sonication for 30 s at 60 Hz, the insoluble residue was removed by centrifugation.

Relative collagen synthesis was estimated by the collagenase assay described by Peterkofsky and Diegelmann (18) with the following modifications. The sonicated material was denatured at 100°C for 10 min. before protein precipitation. The incubation buffer was 50 mM Tris-HCl, pH 7.4, 10 mM CaCl₂, 5 mM N-ethylmaleimide, 1 mM PMSF. Three aliquots were treated with 0, 7.5 and 15 units of bacterial collagenase (Advanced Biofactures, Lynbrook, NY; form III). The percentage of total protein synthesized as collagen was calculated using a factor of 4.1 to correct for the relative abundance of proline and hydroxyproline in collagen compared to proline in non collagenous proteins (19). To analyze the types of collagen synthesized, an aliquot of the sonicate was treated with 100 μg/ml of pepsin (Sigma Chemical Co., St. Louis, MO; P-7012) for 3 h at 4°C. The pepsin digest was brought to pH 8.0 with NaOH and dialyzed for 24 h against electrophoresis buffer. The various collagen types were separated by SDS PAGE on slab or rod gels in the buffer system of Laemmli (20) using a 3% stacking gel and a 6% running gel. Some samples were submitted to a delayed reduction (21) with 2-mercaptoethanol. Radioactive bands on slab gels were visualized by fluorographic detection (22, 23). Quantitative determination was carried out on rod gels fractionated into 1-mm slices which were solubilized in 30% H₂O₂ overnight at 56°C and counted in Aquasol (New England Nuclear, Boston, MA).

Type I and III collagens from human skin and type V collagen (α1[V], α2[V], α3[V] chains) from human placenta were used as standards.

Light Microscopy: Bone and skin cell cultures were stained for calcium phosphate salts using the von Kossa method (24) and for alkaline phosphatase activity as described in Sigma technical Bulletin no. 85 (Sigma Chemical Co.).

Scanning Electron Microscopy: Calvariae were fixed for 1 wk at room temperature in 3% gluteraldehyde in 0.15 M cacodylate buffer, pH 7.4, dehydrated and critical-point-dried. Calvariae were then coated with gold and viewed with a Philips 500 scanning electron microscope.

Transmission Electron Microscopy: Osteoblasts cultured on Permanox petri dishes (Lux Scientific Corp., Newbury Park, CA) were fixed in situ for 1 h at 4°C in 2% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After washing overnight at 4°C with buffer containing 7% sucrose, the specimens were postfixed for 1 h at 4°C in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, stained en bloc for 30 min with 0.1% aqueous uranyl acetate. After dehydration, cells were transferred into glass vials, washed with propylene oxide and embedded in Spurr resin (25).

Fibroblasts grown on glass coverslips (Corning Glass Works) were processed following the in situ embedding method of Chien (26). Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination in a Philips EM 400 electron microscope.

For the cytochemical visualization of alkaline phosphatase activity, osteoblasts grown in culture without β-glycerophosphate were fixed in gluteraldehyde as outlined above. After washing with sucrose-containing buffer, cultures were demineralized with 10% buffered disodium EDTA for 4-6 h and processed for electron microscopy by the Yoshiki and Kurahashi modification (27) of the Mayahara et al. method (28). Cells were incubated for alkaline phosphatase at room temperature for 30 min. Substrate was omitted in controls. After incubation, cells were prepared for electron microscopy as outlined above.

RESULTS

Cell Isolation

Scanning electron microscopy examination of calvariae after careful stripping of the periosteum showed a single layer of endocranial osteoblasts covering the bone matrix of the central

portions of the frontal and parietal bones. When glass fragments were placed on this cell layer, one observed that after 24 h, osteoblasts had migrated onto the glass (Fig. 1). Under the culture conditions described, by day 4, multilayers of osteoblasts were present on glass fragments and stayed there upon removal of the fragments. With longer culture periods, cell multilayers behave as a sheet which tore when glass fragments were removed.

Cell multilayers dispersed by scraping and transferred to petri dishes containing the culture medium, attached to the dish surface within 2 h. Subsequently, cell clusters expanded as cells migrated and divided. Cells in the center of clusters were polygonal shaped but fibroblastlike cells were seen at the periphery. After 7 d in culture, ~1-1.5 × 10⁶ cells were obtained from 10 calvariae.

Collagen Synthesis

Collagens synthesized by osteoblasts during a 24-h incubation with [³H]proline were assayed by collagenase digestion of the combined medium and cell layer proteins. The cell layer contained ~20% of the total collagenase-digestible TCA-precipitable protein and 50% of the total TCA-precipitable noncollagen proteins. Osteoblasts, after 7 d in culture devote 11.2 ± 2.9% (mean ± SD, n = 8) of their total protein synthesis to collagen. This level of collagen synthesis was retained up to 14 d in culture, the longest time studied. Control fibroblast cultures synthesized 7.1 ± 1.0% (n = 3) of their total protein as collagen.

The types of collagen produced by the cultured cells were analyzed by SDS PAGE of the pepsin-digested proteins from the medium and cell layer (Fig. 2). The fluorograms consistently showed two major bands co-migrating with standard
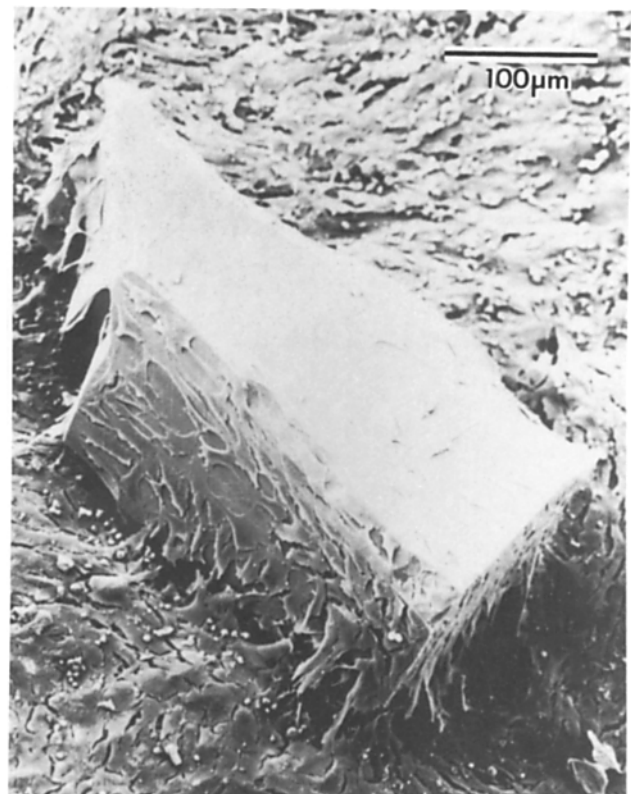

FIGURE 1   Scanning electron micrograph of mouse endocranial osteoblasts which have moved from bone onto a glass fragment after 1 d in culture. × 200.
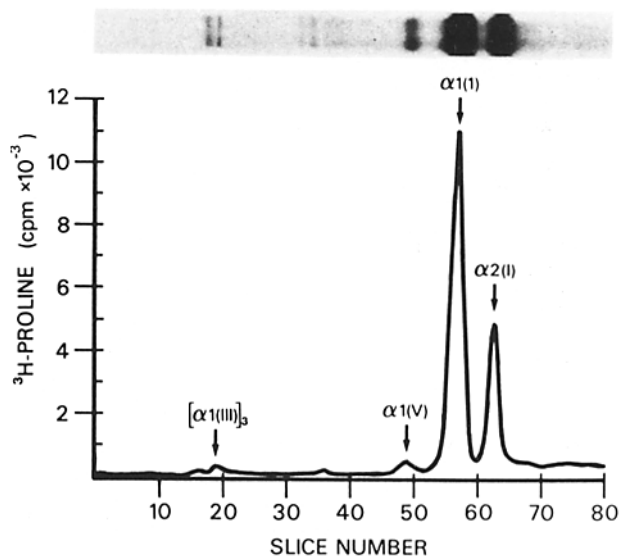
FIGURE 2 SDS PAGE of collagens synthesized by osteoblasts cultured for 7 d. The [³H]proline labeled proteins isolated from the culture medium and cell layer were treated with pepsin, dialyzed and applied on a composite 3% and 6% acrylamide gel. Radioactivity of each 1-mm slice (top) and a corresponding fluorogram (bottom) are shown. The migration positions of the collagen chain standard are indicated. Cross-linked collagen (β and γ components) is present.

$\alpha 1(I)$ and $\alpha 2(I)$ collagen chains. Minor components included β- and γ-chains, type III collagen, identified by its sensitivity to reduction, and a band migrating at the position of human $\alpha 1(V)$ chains. Each of these components represented <3% of the total radioactive collagen and were detected in cultures from day 1 to day 14. All these peptides could be degraded by purified bacterial collagenase.

The $\alpha 1(I)/\alpha 2(I)$ ratio (2.5 ± 0.2, $n = 11$) was closely similar to the ratio found for identically treated collagen from mouse fibroblast cultures (2.4 ± 0.2, $n = 3$). These fibroblasts however synthesized 12–19% of type III collagen.

Mineralization in Osteoblast Cultures

Osteoblast cultures grown in the presence of the alkaline phosphatase substrate, β-glycerophosphate (10 mM, final concentration) showed mineral deposits by 5–6 d. These deposits appeared as white spots on the dish surface and stained positively using the Von Kossa method. Light microscopy of stained sections of cell clusters from these cultures (Fig. 3) showed the presence of cells completely surrounded by the calcified matrix, giving an image of osteocytes.

Electron microscopic examination of these cultures (Fig. 4) revealed the presence in the extracellular space of needle-shaped apatite-like crystals closely associated with striated collagen fibrils and suggested that the initial site of mineralization is associated with extracellular matrix vesicles. Spherical structures similar to the calcification nodules described by several investigators in membranous bone (29–31) were sometimes observed among collagen fibrils.

The same degree of mineralization was obtained in the presence of 5 mM β-glycerophosphate. In contrast, no mineral deposits were found in osteoblast cultures grown in absence of β-glycerophosphate where only banded collagen fibrils and matrix vesicles were present in the extracellular space (Fig. 5).

Mouse skin fibroblast cultures maintained in the presence of 10 mM β-glycerophosphate for up to 14 d did not exhibit any mineral deposits even when plated at very high density where multilayering occured. Extracellular vesicles were not observed in these cultures.

In view of the postulated role of alkaline phosphatase in mineralization, osteoblast cell cultures grown without β-glycerophosphate for 10 d were stained for this enzyme. When examined with the light microscope, the cells in the clusters showed intense staining but some flattened cells away from the clusters failed to stain. In contrast, very few cells stained in fibroblast cultures. At the electron microscopy level, enzyme reaction product was found associated with plasma membrane, collagen fibers and matrix vesicles.

DISCUSSION

The specific function of osteoblasts is to elaborate a calcified matrix. The cells we isolated from newborn mouse calvaria and cultured for up to 14 d have this functional characteristic of osteoblasts.

The technique of isolation used is based on the ability of osteoblasts to migrate from the bone onto glass fragments as demonstrated by Jones and Boyde (17). This method avoids the use of digestive enzymes which might cause cellular damage including loss of membrane hormone receptors. The cells which populate the glass fragments lie in a confluent monolayer on the bone matrix they have synthesized and are fully differentiated osteoblasts. Osteoprogenitor cells have been removed by the dissection procedure and contamination of the cell population by osteoclasts and chondrocytes is likely to be reduced to very small proportions as these two cell types are found in marginal sutural areas well away from the glass fragments. Although there is no objective marker to assess the homogeneity of our cell preparations, we believe that the cells are mainly osteoblasts because of the collagen phenotype expressed and their osteogenic capacity.

It is interesting to note that the osteoblasts after separation from bone are able to divide under culture conditions. We do not know whether this implies a dedifferentiation into precursor type cells which can proliferate and differentiate again into osteoblasts.
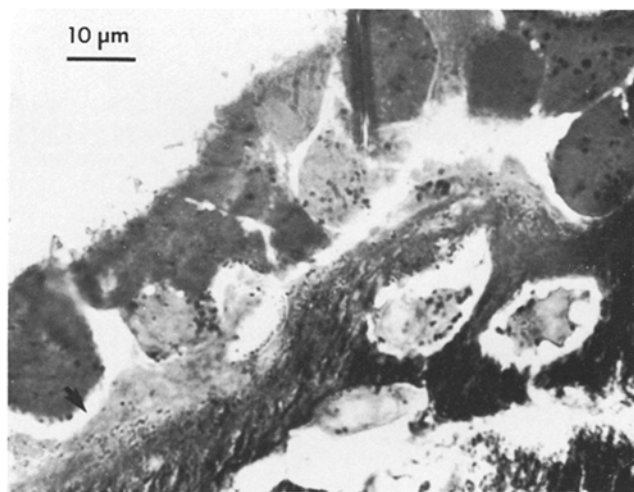

FIGURE 3 Light micrograph of a Von Kossa–Toluidine Blue O stained 1-μm thick section from a cell cluster grown in presence of 10 mM β-glycerophosphate for 10 d. In the dark stained mineralized matrix are included cells analogous to osteocytes. The cuboid cells, on the periphery, cover nonmineralized osteoid (arrow) and appear similar to osteoblasts. × 1,125.
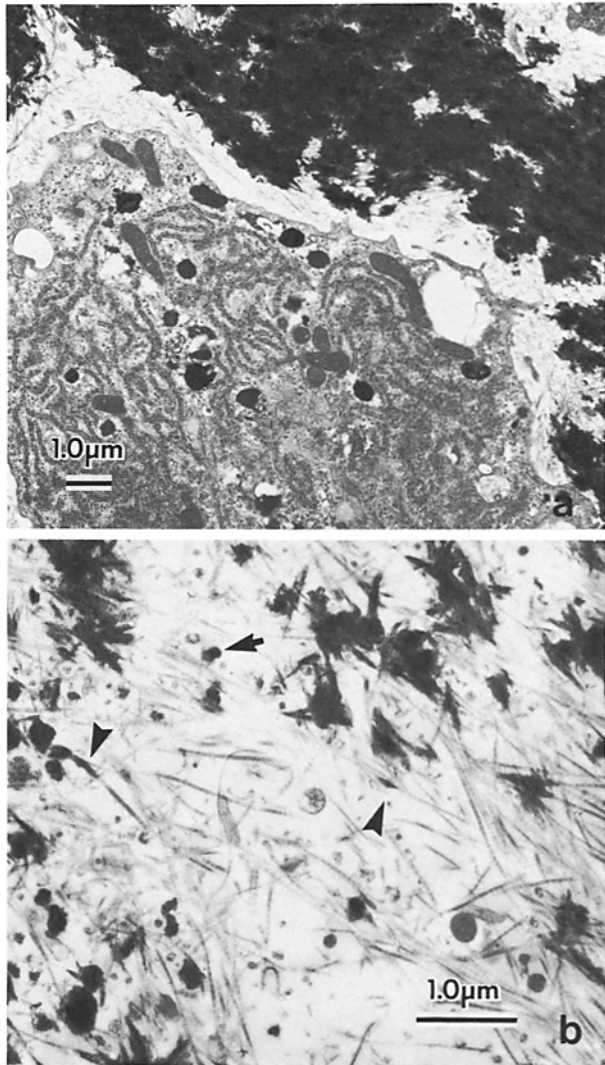
FIGURE 4 Electron micrograph of the extracellular matrix surrounding an osteoblast cultured for 10 d in presence of 10 mM β-glycerophosphate. An osteoid layer separates the cell from the mineralized matrix which contains needle-shaped apatite-like crystals (a). × 5,900. In the extracellular space, matrix vesicles, some containing apatite-like crystals (arrow) are present. Crystals appear closely associated with banded collagen fibrils (arrowheads) (b). × 13,200.

Normal bone matrix is considered to contain only type I collagen (32). The isolated cells in culture synthesized primarily type I collagen (~95%). This collagen phenotype was stable throughout the culture time studied (up to 14 d after cell separation from the glass fragments) and was independent of cell density. As the ratio of $\alpha 1(I)$ to $\alpha 2(I)$ collagen chains was close to the expected 2:1 ratio and as a similar ratio was found for fibroblast cultures, the presence of sizable amounts of type II collagen in the osteoblast cultures can be ruled out. The slightly elevated ratio of $\alpha 1$ to $\alpha 2$ may reflect the presence of type I trimer. Whether the synthesis of small amounts of type III and type V collagens by the isolated cells reflects the collagen phenotype in vivo is difficult to assess. These components may not be detectable biochemically at these levels. Recently, traces of type V collagen were reported to be present in human fetal bone (33). The collagen phenotype we observed is similar to that reported for cultured clones of fetal rat calvarial cells (12). Synthesis of type III and type V collagens have been reported respectively in osteoblast-like cell cultures from mouse (14) and chick (34) calvariae.

The isolated osteoblasts retain in culture their ability to initiate mineral deposition. This mineral deposition is not due to a physicochemical precipitation of calcium phosphate but is a cell related process for the following reasons: (a) the observed pattern of mineralization (Fig. 4) closely resembled that seen in vivo by the presence of an osteoid layer surrounding the cells and separating them from the mineralized matrix and by the presence of extracellular vesicles and calcification nodules which are believed to play a role in the process of calcification (31, 35); (b) skin fibroblasts cultured under identical conditions (up to 14 d in presence of 10 mM β-glycerophosphate) failed to exhibit mineral deposits detectable by light and electron microscopy, even when they grew in multicellular layers. This last finding is in contrast with the report of Williams et al. (11) who described mineralization in osteoblast and fibroblast cultures derived from adult rat calvariae and skin respectively.

Very recently, osteoblast-like cells isolated from calvariae of embryonic chicks (36) and newborn mice (37) and kept in culture for several days were reported to undergo calcification when implanted in an "in vivo" environment. However, these cells never formed a calcified matrix in vitro.
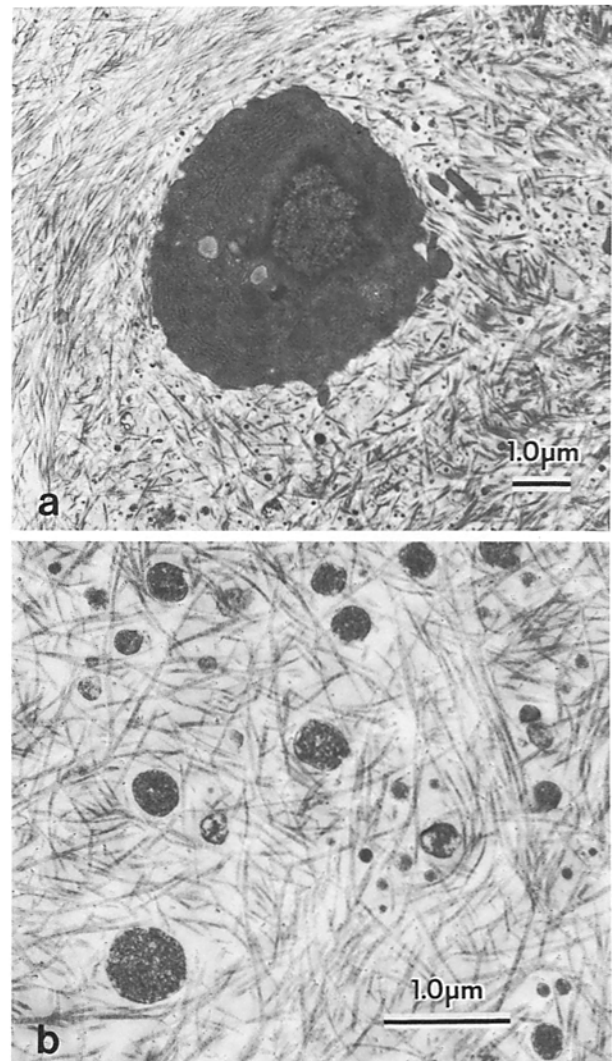

FIGURE 5 Electron micrograph of a 10-d osteoblast culture maintained in absence of β-glycerophosphate. No mineral deposits are found in the extracellular space (a) × 7,600. This space contains numerous vesicles of variable size and striated collagen fibrils (b). × 16,600.

Apart from osteoblast-like cell cultures, periosteal cells, either isolated (38) or in organ culture (15, 16, 39), and mesenchymal cells (40, 41) derived from embryonic bone were shown to express osteogenic capacity in culture.

Appropriate levels of organic phosphate were required for mineralization to occur in culture, as demonstrated recently by Tenenbaum and Heersche (16) using cultured chick periostea. No mineralization took place in the cultures when the medium inorganic phosphate concentration was increased from 0.9 mM (in DME) to 3 mM, corresponding to the serum phosphate concentration of the normal mouse. In the presence of 5 or 10 mM β-glycerophosphate, mineral deposits were reproducibly found associated to each cell cluster from day 5-6 of the cultures.

It is interesting to note that the cells within the clusters are those which stained intensely for alkaline phosphatase activity. The precise relationship between alkaline phosphatase and calcification is a source of controversy. Many functions have been postulated for alkaline phosphatase such as a role in the transfer of phosphate groups from cells to matrix and in the removal of inhibitors of mineralization (for a review, see reference 42). The enzyme is known also to cleave phosphate bonds and it is possible that the presence of β-glycerophosphate in the medium may allow an adequate inorganic phosphate concentration to be present at sites of mineralization (43). In this respect, our ultrastructural observation of alkaline phosphatase activity in matrix vesicles, structures which seem associated with the initial mineralization sites (35) suggests a role for alkaline phosphatase in the process of mineralization.

Apart from organic phosphate concentration, there is another factor that influences the onset of mineralization in vitro. Our experiments with primary cultures and subcultured cells (data not shown) suggest that mineralization only occurs in multilayer areas. The reason for this is not clear. Compaction and cell interactions may be required to obtain an adequate environment at mineralizing sites.

In conclusion, the cells we isolated and cultured for up to 14 d were able to elaborate in vitro a calcified type I collagen matrix, a function specific to the osteoblast cell line. This cell culture system should therefore allow the study of the mechanisms involved in matrix mineralization.

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