

In Vitro Differentiation and Calcification in a New Clonal Osteogenic Cell Line Derived from Newborn Mouse Calvaria

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ABSTRACT We investigated the capacity of a clonal osteogenic cell line MC3T3-E1, established from newborn mouse calvaria and selected on the basis of high alkaline phosphatase (ALP) activity in the confluent state, to differentiate into osteoblasts and mineralize in vitro. The cells in the growing state showed a fibroblastic morphology and grew to form multiple layers. On day 21, clusters of cells exhibiting typical osteoblastic morphology were found in osmiophilic nodular regions. Such nodules increased in number and size with incubation time and became easily identifiable with the naked eye by day 40–50. In the central part of well-developed nodules, osteocytes were embedded in heavily mineralized bone matrix. Osteoblasts were arranged at the periphery of the bone spicules and were surrounded by lysosome-rich cells and a fibroblastic cell layer. Numerous matrix vesicles were scattered around the osteoblasts and young osteocytes. Matrix vesicles and plasma membranes of osteoblasts, young osteocytes, and lysosome-rich cells showed strong reaction to cytochemical stainings for ALP activity and calcium ions. Minerals were initially localized in the matrix vesicles and then deposited on well-banded collagen fibrils. Deposited minerals consisted exclusively of calcium and phosphorus, and some of the crystals had matured into hydroxyapatite crystals. These results indicate that MC3T3-E1 cells have the capacity to differentiate into osteoblasts and osteocytes and to form calcified bone tissue in vitro.

Recently, we established eight cell lines from newborn mouse calvaria and selected a clone, designated MC3T3-E1, from one of these lines on the basis of its high alkaline phosphatase (ALP) activity in the resting state (1). Cells of this clonal line had very low ALP activity in the growing state, but enzyme activity increased several hundredfold after cultures reached a confluent state. The cells produced abundant fibrous intercellular substances. In addition, intercellular spaces in day-30 cultures stained positively with alizarin red S. These observations suggest that MC3T3-E1 cells are an osteogenic cell line, having the capacity to differentiate into osteoblasts and deposit minerals in vitro.

We undertook the studies described here to further characterize the process of differentiation of MC3T3-E1 cells and calcification in vitro. We found that calcified bone tissue was formed in MC3T3-E1 cultures by a process closely resembling that observed in intramembranous osteogenesis in vivo. Mineral deposits were identified as hydroxyapatite by energy dispersive x-ray analysis and electron diffraction analysis.

MATERIALS AND METHODS

Cell Culture: The MC3T3-E1 cells were grown in alpha modification of Eagle's minimal essential medium (α -MEM) (Flow Laboratories, McLean, VA) supplemented with 10% newborn calf serum (Flow Lab., Stanmore, New South Wales, Australia) and 60 μ g/ml of kanamycin sulfate (Meiji Seika, Tokyo, Japan). Cells were maintained at 37°C in a fully-humidified atmosphere of 5% CO₂ in air and subcultured every 3 d by dissociating with 0.001% pronase E (Kaken Kagaku, Tokyo, Japan) plus 0.02% EDTA in Ca²⁺- and Mg²⁺-free PBS. For experiments, cells in logarithmic growth phase were dissociated, counted in a hemocytometer, and inoculated at 5 × 10⁴-cells/35-mm plastic dish (Falcon Labware, Oxnard, CA) containing 1.5 ml of medium. They were cultured as mentioned above and fed every 3 d.

Electron Microscopy: Cultures were fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature after washing twice with the same buffer. They were rinsed with the buffer and postfixed with 1% osmium tetroxide in the buffer for 2 h at 4°C. After dehydration in graded ethanols, they were embedded in Quetol 812 (Nissin EM, Tokyo). The blocks were sectioned with diamond or glass knives using an Ultratome III 8800 (LKB Instruments, Inc., Bromma, Sweden). Thick sections for light microscopy were stained with 1% toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a

JEM-100C electron microscope. Electron diffraction analysis was carried out using unstained sections.

Cytochemical Staining for ALP Activity: For light microscopy, fixed cultures were stained for 30 min at 37°C using naphthol AS-MX phosphate as a substrate and fast red violet LB salt as a coupler (2). Some cultures were frozen, cut in 10- μ m sections with a cryostat, and similarly stained. For electron microscopy, frozen sections (40–50 μ m thickness) of prefixed cultures were incubated for 30 min at 37°C in a medium containing 20 mM Na β -glycerophosphate, 3.9 mM magnesium sulfate, 2 mM lead citrate, and 28 mM Tris-maleate (pH 8.3) (3). Then they were processed in the same way as described above. As a control, sections incubated in the absence of the substrate were prepared.

Localization of Calcium Ions: Cultures were rinsed with 0.25 M sucrose and fixed with 2.5% glutaraldehyde in 0.1 M K-phosphate buffer (pH

7.4) containing 2% potassium pyroantimonate for 1 h at room temperature as described by Zadunaisky (4). After being rinsed with 0.25 M sucrose, they were postfixated with 1% osmium tetroxide in the phosphate buffer for 2 h at 4°C. Then they were processed as described above. Cultures decalcified with 5% EDTA for 2 h at 4°C before postfixation were used as a control.

Energy Dispersive X-ray Analysis: Unstained sections (150–400 nm thickness) were mounted on copper grids and coated with carbon. These sections were quantitatively analyzed according to the method of Cliff and Lorimer (5) using a H-600H electron microscope (Hitachi, Hitachi, Japan) fitted with a Kevex-7,000 Q energy dispersive x-ray analysis system (Kevex Corp., Foster City, CA). All analyses were carried out at 100 kV for 100 s with a probe current of 1×10^{-9} A. Synthetic hydroxyapatite crystals were generously provided by Dr. H. Aoki (Tokyo Medical and Dental University, Tokyo) and used as a standard. The *K* value for phosphorus relative to calcium in the equation of Cliff and Lorimer (5) was determined from characteristic x-ray intensities of phospho-

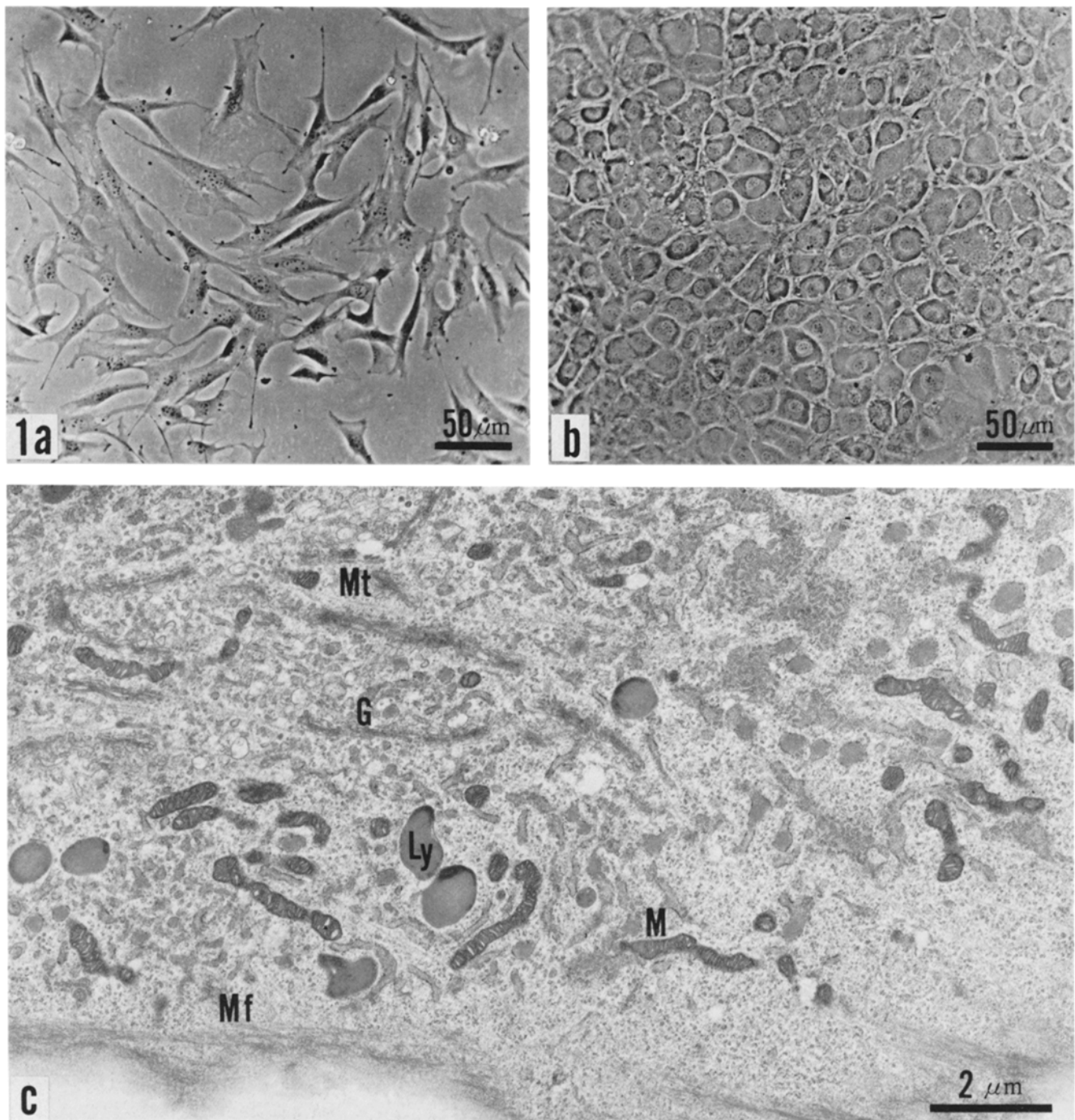


FIGURE 1 Phase-contrast microscopic appearance of MC3T3-E1 cells in day-2 (a) and day-4 (b) cultures, and ultrastructure of the cytoplasm of a cell in day-2 culture, sectioned parallel to the surface of the culture dish (c). Numerous microfilaments (Mf), microtubules (Mt), mitochondria (M), Golgi apparatus (G), and lysosomes (Ly) are observed. (a and b) $\times 250$. (c) $\times 10,000$.

rus and calcium in the standard. P/Ca ratios in the sections were determined using a K value of 1.813.

RESULTS

Bone Tissue Formation by MC3T3-E1 Cells

MC3T3-E1 cells in logarithmic growth phase showed a fibroblastic morphology, containing abundant microtubules and microfilaments in their cytoplasm; but the Golgi apparatus and rough endoplasmic reticulum were not so extensive (Fig. 1*a* and *c*). The cells grew with a population doubling time of ~18 h. On day 4 of culture, when the cultures reached a confluent monolayer at a density of $5-6 \times 10^4$ cells/cm², their cellular outlines had a mosaic appearance (Fig. 1*b*). Then the cells continued to grow slowly and formed multiple cell layers (Fig. 2*c*).

A few collagen fibrils, which increased both in number and diameter as the culture period lengthened, were identified around the actively growing cells. By day 18, the banding pattern of such fibrils became evident, exhibiting a 60-70-nm axial periodicity similar to that of bone tissue collagen (Figs. 3*b*, 4*e*, and 5).

Small osmiophilic nodular regions were first identified in day-21 cultures. In these nodules, clusters of small cells were surrounded by a dense fibrous matrix (Fig. 6*a*). The cells exhibited ultrastructural characteristics of typical osteoblasts (Figs. 3*a* and *b* and 6*c*). The nodules increased in number and size with incubation time, and some of them fused with each other. The central part of more developed nodules was occupied by young osteocytes, and mineral deposition was detected in the bone matrix (Fig. 6*b* and *c*). Osteoblasts were arranged at the periphery of the bone spicules which were enveloped by a periosteumlike cell layer (Fig. 6*b* and *c*). Several layers adjacent to the osteoblast layer consisted of cells containing numerous electron-dense lysosomes and myelinlike figures, as

well as well-developed Golgi apparatus. Cells near the osteoblasts consistently showed, in the many sections observed, an intermediate morphology between lysosome-rich cells and osteoblasts. By day 40-50, the nodules became easily identifiable, without staining, as white spots with the naked eye and as dark-brown protuberant areas by microscopy (Fig. 2*a*). At this stage, osteocytes were completely embedded in a heavily mineralized matrix (Figs. 2*b* and *c* and 3*c*). Some of the osteocytes in the central part had deteriorated, probably due to insufficient diffusion of nutrients.

Ultrastructural Observation of Mineral Deposition in the Bone Tissue

In day-24 cultures, a large number of membrane-bounded vesicles were found around the osteoblasts and young osteocytes (Figs. 3*a*, 4*a-d*, 5, 6*c*, 7*b*). The matrix vesicles were usually round or ovoid, and only rarely irregular in shape, varying in diameter from 60 to 400 nm. Then vesicles filled with needle-shaped crystals increased in number. The crystals were deposited on an organic matrix and formed clusters (Fig. 4*b* and *d*). By day 30, the crystals had become arranged alongside the collagen fibrils, forming large calcified spherules (Figs. 4*e*, 5, and 6*c*). Consequently, the osteocytes were encompassed by mineralized materials, and an extracellular space separated the cell surface from the mineralized layer (Fig. 3*c*). These observations demonstrate that, in MC3T3-E1 cultures, minerals are initially localized in matrix vesicles and that this is followed by additional calcification of the organic matrix, in a fashion similar to what has been observed *in vivo* (6).

Cytochemical Observations

To further examine the functional roles of osteoblasts, osteocytes, and matrix vesicles in the calcification process in our cultures, we employed cytochemical procedures. Most cells in

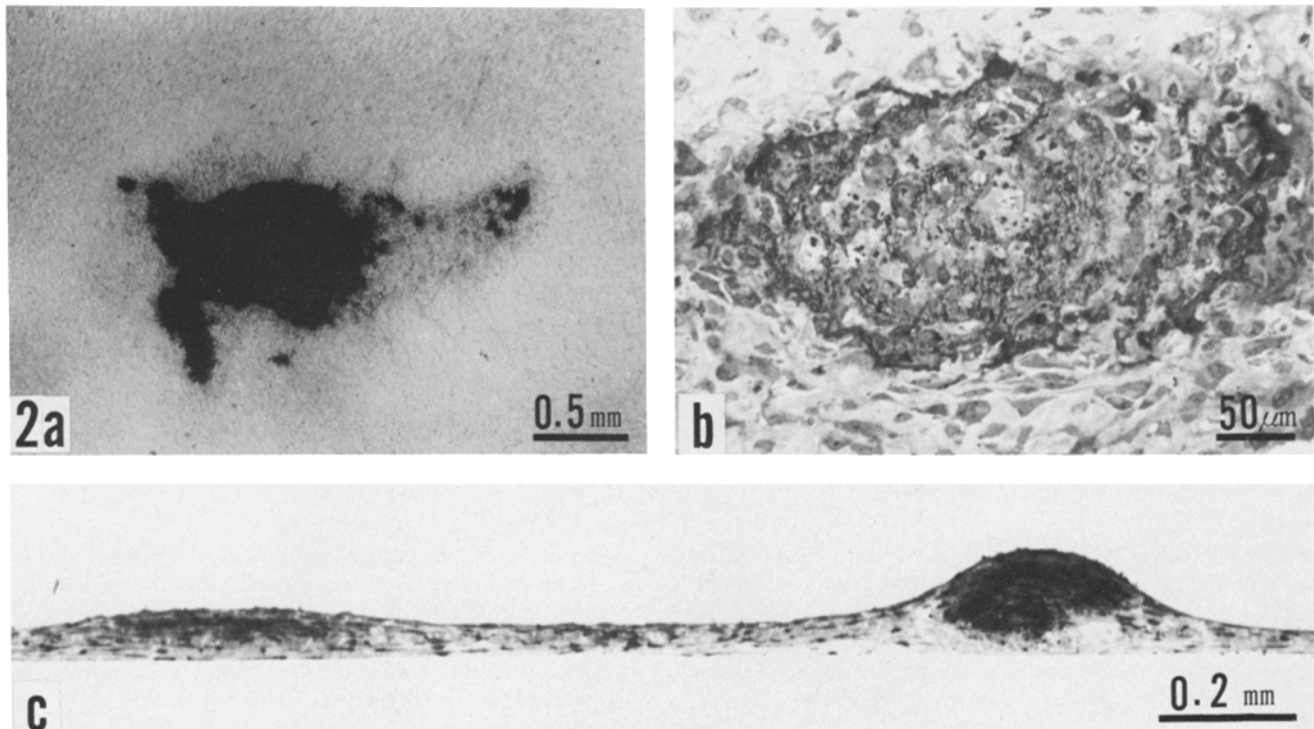


FIGURE 2 Light micrographs of highly mineralized nodules in day-45 cultures. (a) Unstained culture. $\times 150$. (b) A horizontally sectioned nodule. $\times 210$. (c) A vertically sectioned nodule. Osteocytes are embedded in a highly mineralized intercellular substance. Some of them in the central part have become necrotic. $\times 90$.

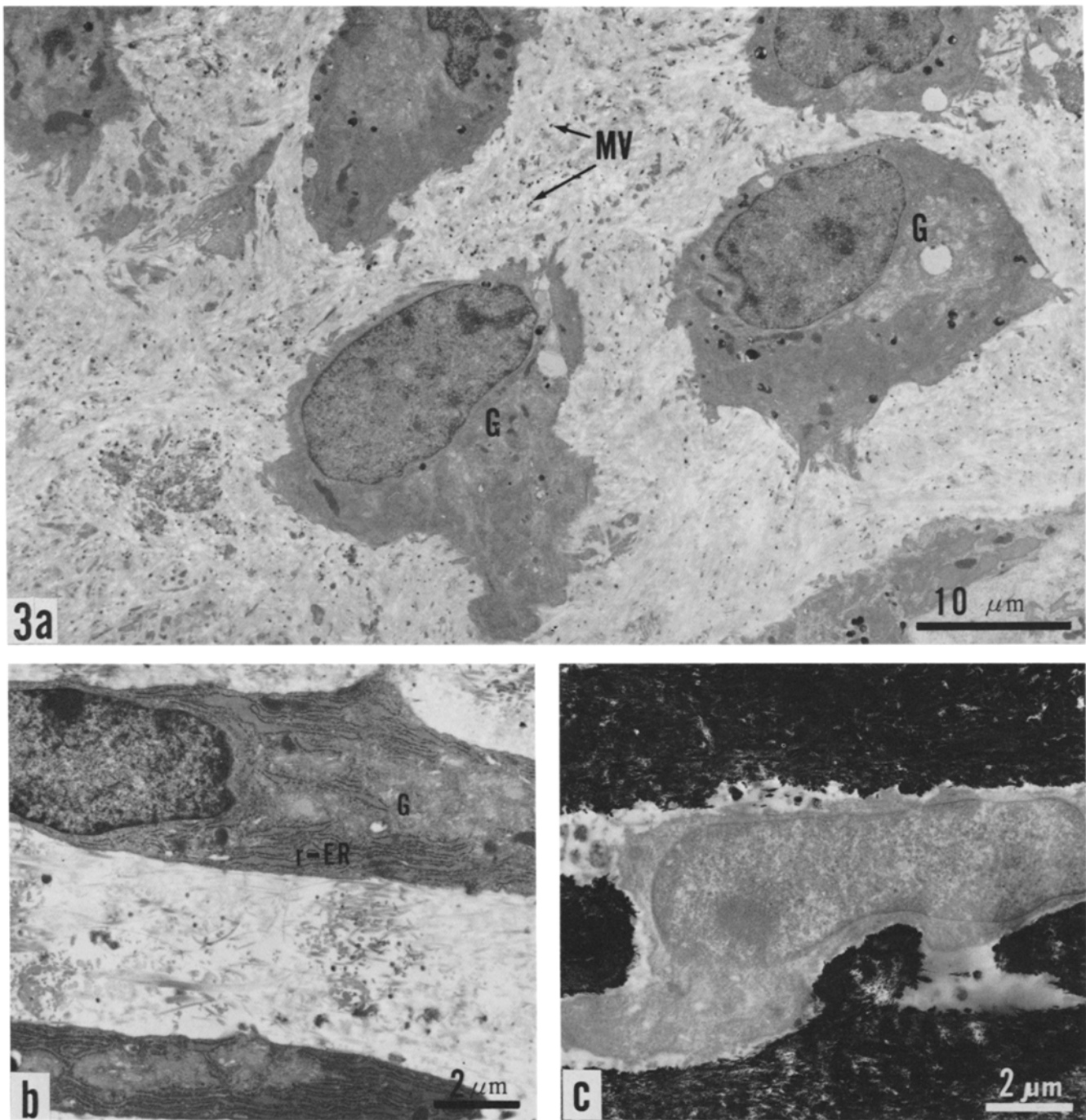


FIGURE 3 Electron micrographs of osteoblasts and osteocytes in day-45 cultures. Horizontally (a) and vertically (b) sectioned osteoblasts are seen. The cells contain parallel arrays of rough endoplasmic reticulum (*r-ER*), and the Golgi apparatus (G) is located eccentrically to the nucleus. Numerous matrix vesicles are scattered throughout the collagenous matrix. (c) A vertical section of an osteocyte. The cell has decreased cytoplasmic volume and organelle content and it completely surrounded by a heavily calcified intercellular substance. (a) $\times 2,500$. (b and c) $\times 7,000$.

the nodules showed extremely high ALP activity (Fig. 7a). As shown in Fig. 7b, strong reaction products were observed along the plasma membranes of osteoblasts and in matrix vesicles. In addition, plasma membranes of young osteocytes and lysosome-rich cells were also intensely stained, whereas aged osteocytes were negative (data not shown), as observed in vivo (7, 8).

When sections fixed in the presence of potassium pyroantimonate were examined, granular electron-dense precipitates of calcium pyroantimonate were found mainly in the matrix

vesicles and along the plasma membranes of osteoblasts, young osteocytes, and some lysosome-rich cells (Figs. 5 and 6c). Fine precipitates were also detected in Golgi apparatus and mitochondria as previously reported in vivo (9, 10). The presence of calcium and antimony in the precipitates was confirmed by energy dispersive x-ray analysis (data not shown). However, such precipitates were scarcely found in undifferentiated cells or aged osteocytes. These observations show that MC3T3-E1 cells express differentiated functions during their differentiation in culture and play an active role in mineralization.

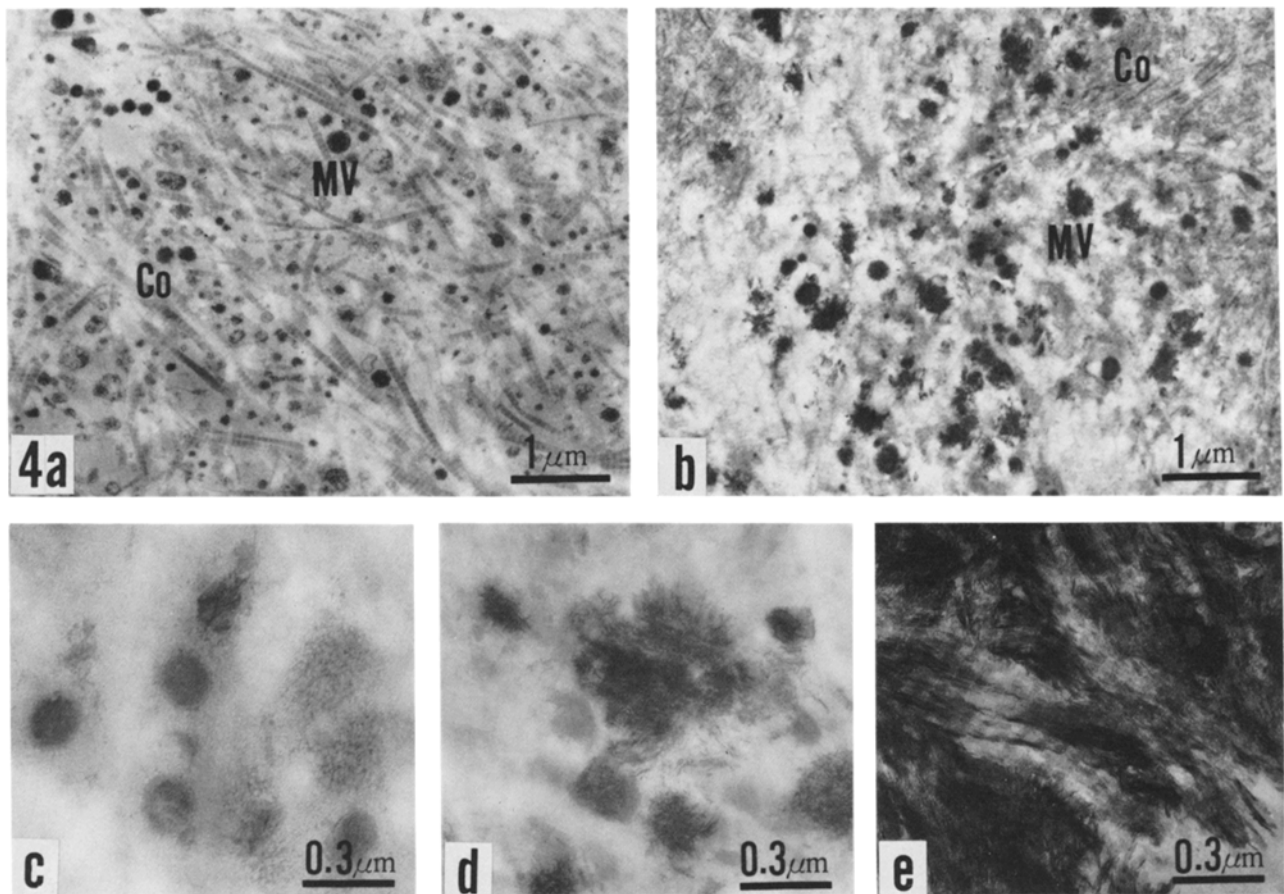


FIGURE 4 Deposition of minerals in MC3T3-E1 cultures. (a) Matrix vesicles (MV) among collagen fibrils (Co) in a day-24 culture. (b) Matrix vesicles containing varying amounts of minerals in a day-24 culture. (c) High magnification view of the matrix vesicles in a. Trilamellar membrane structure of the matrix vesicles is evident. (d) High magnification view of the matrix vesicles and a cluster of needle-shaped crystals in b. (e) Minerals deposited on well-banded collagen fibrils in a day-30 culture. (a and b) $\times 13,000$. (c, d, and e) $\times 40,000$.

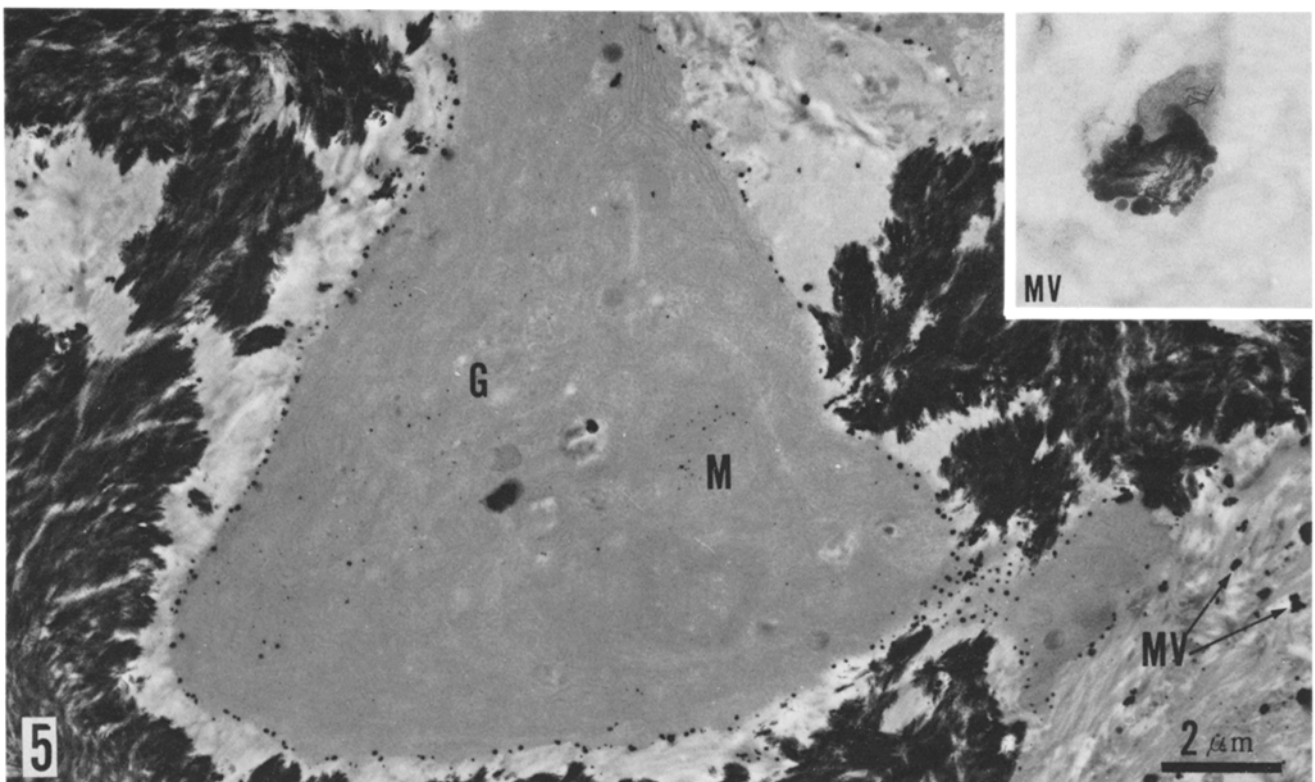


FIGURE 5 Cytochemistry for calcium ion detection. Precipitates of calcium pyroantimonate are detected on the surface of the plasma membrane of a young osteocyte and matrix vesicles (MV). Fine reaction products are also seen in the mitochondria (M) and Golgi apparatus (G). (Inset) High magnification view of calcium pyroantimonate precipitated in the matrix vesicles. $\times 8,000$.

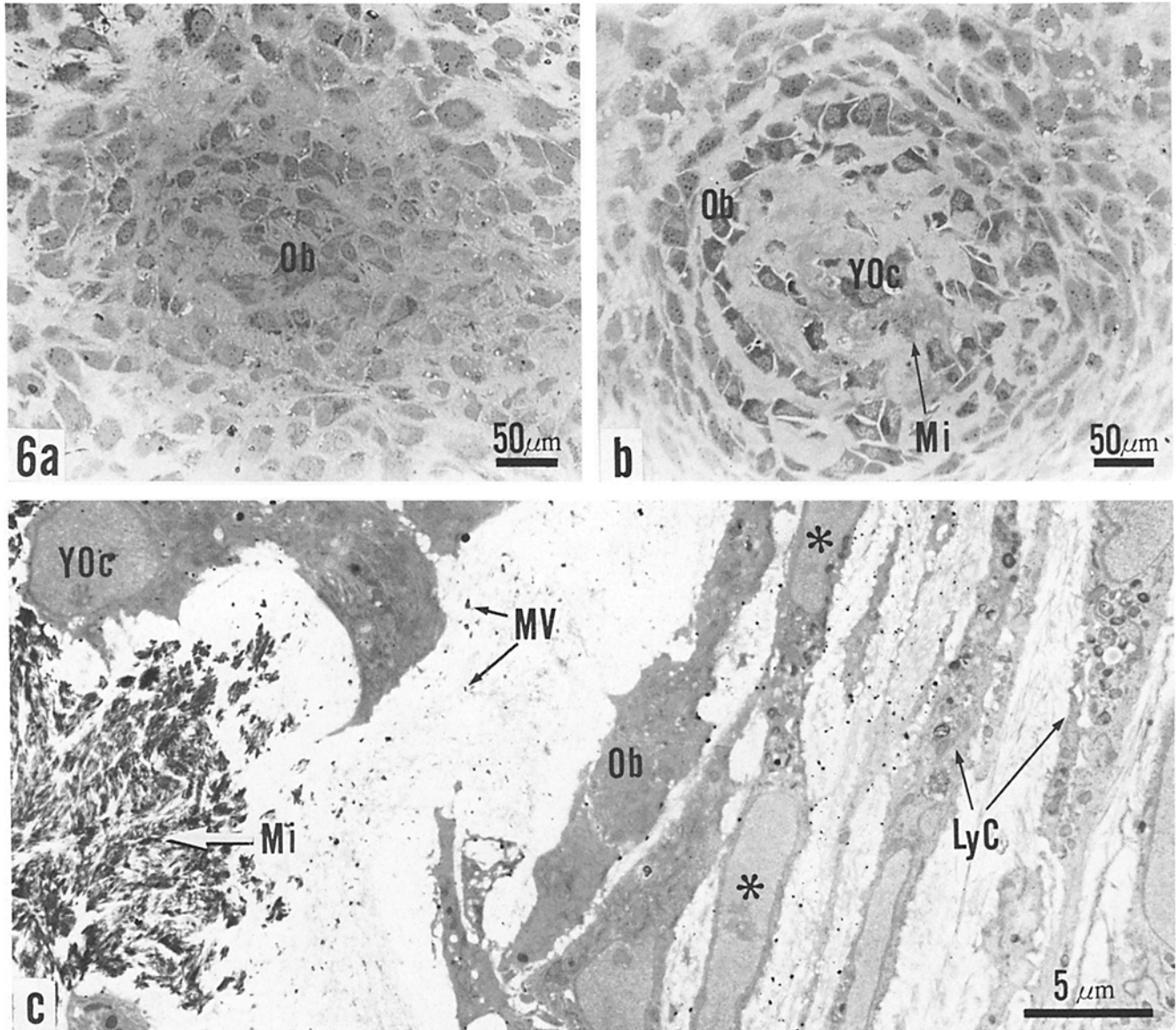


FIGURE 6 Nodule formation in MC3T3-E1 cell cultures. Osmiophilic areas were sectioned parallel to the culture dish surfaces. (a) A cluster of osteoblasts (*Ob*) surrounded by dense fibrous matrix in a day-21 culture. $\times 180$. (b) A more developed nodule in a day-24 culture. Young osteocytes (*YOc*) are embedded in slightly mineralized bone matrix (*Mi*) and are surrounded by an osteoblast layer. $\times 180$. (c) Electron micrograph of a nodule in a day-30 culture. Young osteocytes embedded in mineralized matrix, the osteoblast layer, and a periosteumlike cell layer are observed. Within the periosteumlike layer, several layers adjacent to the osteoblasts consist of lysosome-rich cells (*LyC*). Two intermediate type cells (*) between lysosome-rich cells and osteoblasts are also identifiable. Since this section was fixed in the presence of potassium pyroantimonate, precipitates of calcium pyroantimonate are detected in the matrix vesicles (*MV*) and along the plasma membranes of osteocytes, osteoblasts, and some lysosome-rich cells. $\times 3,800$.

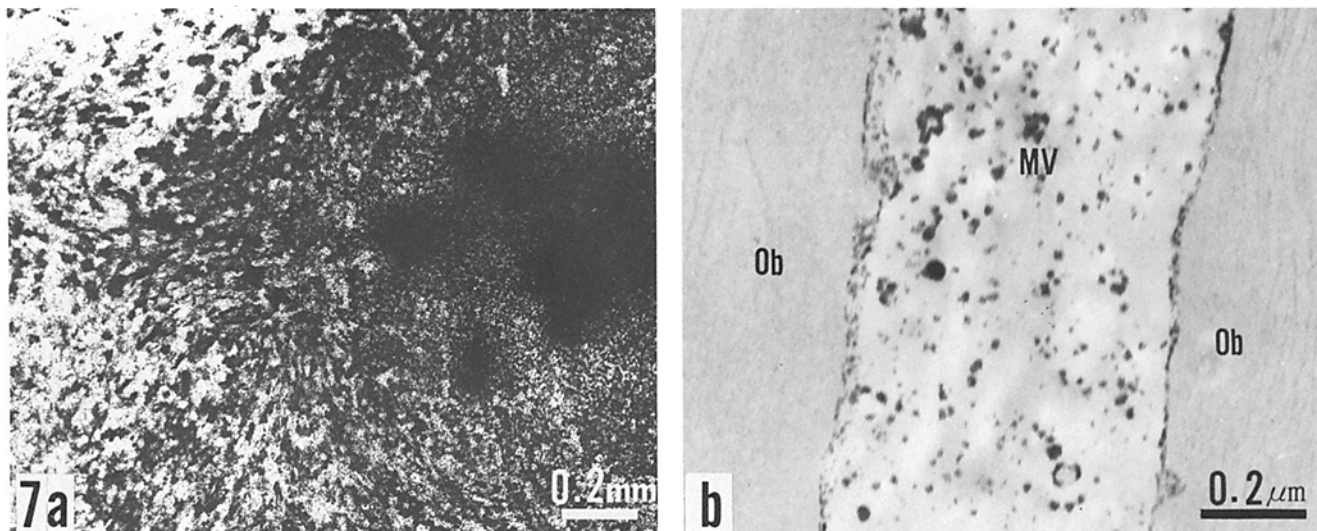


FIGURE 7 Cytochemistry of ALP activity. (a) A light micrograph of a nodule. $\times 65$. (b) An electron micrograph showing intense reaction products on the plasma membrane of osteoblasts (*Ob*) and matrix vesicles (*MV*). $\times 28,000$.

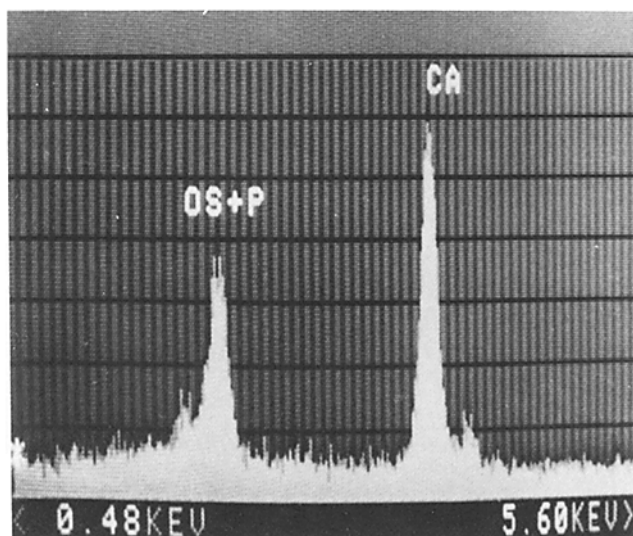


FIGURE 8 X-ray energy spectrum of highly mineralized area. Prominent peaks of Ca- K_{α} , K_{β} , and P-K, in addition to Os-M, are identifiable.

Analysis of Crystals Deposited in the Bone Tissue

Elemental components and states of maturation of the crystals deposited in our cultures were determined using energy dispersive x-ray analysis and electron diffraction analysis. As shown in Fig. 8, prominent peaks for calcium and phosphorus, in addition to a peak due to osmium which was introduced during the fixation, were detected in the profiles of the mineralized regions. P/Ca ratios of the crystals were close to the 0.6 value of hydroxyapatite (0.51–0.67). P/Ca ratios of the crystals in the matrix vesicles tended to be <0.6 . Minimal amounts of calcium and phosphorus were detected in the unmineralized matrix (data not shown). Furthermore, typical electron diffraction patterns of the crystals exhibited that of nonoriented hydroxyapatite (Fig. 9a and b). These results indicate that crystals deposited in MC3T3-E1 cultures consist exclusively of calcium and phosphorus, and some of them have matured into hydroxyapatite crystals.

DISCUSSION

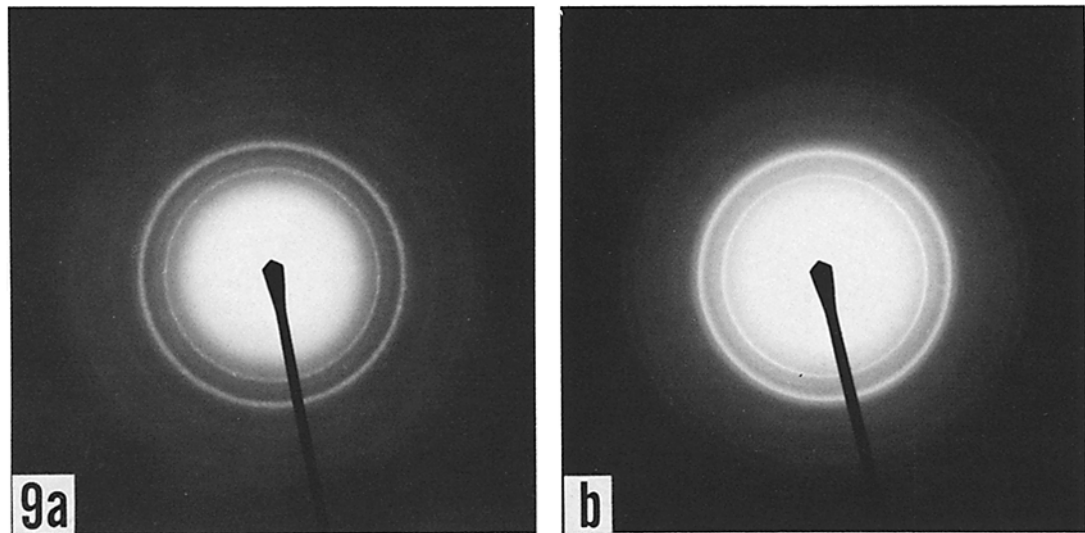
Mineral deposition has been demonstrated in only a few *in vitro* cell culture systems (11–17). The primary culture system of fetal rat calvaria cells developed by Binderman et al. (11, 12) had been the sole successful system of mammalian bone cells. Recently, Williams et al. (16) reported that cells isolated from adult cat calvaria and several clones derived from them retained the ability to mineralize *in vitro* even after extended passages. However, they could not observe a consistent association between mineralized regions and collagen fibrils. Aubin et al. (18) also isolated several hormone-sensitive clones from fetal rat calvaria cells, but they did not refer to the ability of such clones to mineralize *in vitro* (18). We established a clonal cell line, MC3T3-E1, from newborn mouse calvaria, exhibiting high ALP activity in the resting state (1). The present results demonstrate that these MC3T3-E1 cells have the capacity to differentiate into osteoblasts, to deposit hydroxyapatite in well-developed bone matrix, and to further express the differentiated state of osteocytes.

Actively growing MC3T3-E1 cells appear to be at the stage of osteoprogenitor cells (19), since they showed a typical fibroblastic morphology, had very low ALP activity (1), and had the capacity to differentiate into osteoblasts. MC3T3-E1 cells were established under a rigid 3T3 subculture schedule (1), which is known to select for cells sensitive to postconfluence inhibition of division (20). Nevertheless the cells grew to form multiple cell layers. This appears to be due to their active secretion of a collagenous intercellular substance, since they stopped growing at the monolayer stage when collagen production was prevented under ascorbic acid-deficient conditions (H. Sudo and H. Kodama, unpublished observations).

Cells differentiated into osteoblasts were initially identified as clusters in localized areas. Such areas increased in number and size and developed into calcified bone tissue. The histogenic processes involved resemble those observed at ossification centers in membrane bones *in vivo* (21) which suggests that the expression of the developmental program is initiated in only a limited number of cells. However, nothing is known about the nature of the stimulus or stimuli.

Lysosome-rich cells were found in the periosteumlike cell layer. These cells are probably similar to those which have

FIGURE 9 Electron diffractograms of synthetic hydroxyapatite crystals (a) and crystals deposited in MC3T3-E1 cultures (b). The latter shows typical reflections of nonoriented hydroxyapatite crystals.



been observed in vivo (7) and in the primary culture of bone cells (11). We found such cells in cultures of a clonal cell line, and they were consistently located adjacent to osteoblasts; also, cell types intermediate between lysosome-rich cells and osteoblasts lay contiguous to the osteoblasts. These observations suggest that these lysosome-rich cells are immediate precursors of osteoblasts.

Various morphological parameters, along with supportive cytochemical observations, suggest that mineral deposition in our system proceeds in a manner similar to that which has been observed in a variety of systems in vivo, and that osteoblasts, young osteocytes, and matrix vesicles play active functional roles in the process of mineralization. Our cell line MC3T3-E1 is an established clonal osteogenic cell line, and thus it should be a useful model system for studies of differentiation of bone cells and the mechanism(s) of biological calcification.

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