FORMATION OF BONE TISSUE IN CULTURE
FROM ISOLATED BONE CELLS

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ABSTRACT
A system is described for the formation of bone tissue in culture from isolated rat bone
cells. The isolated bone cells were obtained from embryonic rat calvarium and periosteum or from traumatized, lifted periosteum of young rats. The cells were cultured for a
period of up to 8 wk, during which time the morphological, biochemical, and functional
properties of the cultures were studied. Formation of bone tissue by these isolated bone
cells was shown, in that the cells demonstrated osteoblastic morphology in light and
electron microscopy, the collagen formed was similar to bone collagen, there was minerali-
zation specific for bone, and the cells reacted to the hormone calcitonin by increased cal-
cium ion uptake. Calcification of the fine structure of the cells and the matrix is described.
Three stages in the calcification process were observed by electron microscopy. It is con-
cluded that these bone cells growing in vitro are able to function in a way similar to such
cells in vivo. This tissue culture system starting from isolated bone cells is therefore suitable
for studies on the structure and function of bone.

INTRODUCTION
Various theories of mammalian bone calcification
have been proposed, each of them based on bio-
logical and biophysical phenomena’s taking place
in living bone (1). Calcification in bone tissue is
preceded by the formation of an extracellular
organic matrix, primarily secreted by bone cells
(2). The bone-forming cells, active osteoblasts
and osteocytes, control the synthesis of this extra-
cellular matrix and regulate the exchange be-
 tween ions present in the bulk extracellular fluids
and those present in mineralizing bone collagen
(3).

Recent evidence established the role of bone
cells in forming functional units that are vitally
concerned with calcium homeostasis (4). It is
therefore of importance for the study of the bio-
chemical and biophysical aspects of living bone to
develop a model system of living osteoblasts in
which differentiation, secretion of bone collagen,
and calcification can proceed.

After the pioneering work of Fell (5), few
attempts have been reported in the literature to
grow bone cells capable of forming bone collagen
in tissue culture. Most of these studies were done
in organ cultures in which whole bone or frag-
ments of bones were cultured and were analyzed
histologically and biochemically (6, 7). A method
for culturing isolated bone cells was reported by
Rose (8), using a special multipurpose culture
chamber. The osteogenic capacity of embryonic
chicken fibroblasts in tissue culture was demon-
strated by Fitton-Jackson (9). Bard and his
coworkers (10) recently reported the isolation of bone cells from various sources. The cells, however, were kept for a short time only and did not undergo calcification. At the same time, morphological and histochemical evidence suggests that the functional activity of bone cells is correlated to their ultrastructure (11, 12). In these studies both mineralized (13) and decalcified (14) bone specimens taken from living tissue have been used. Nevertheless, the various steps involved in bone formation from the deposition of the extracellular matrix up to its calcification could not be investigated in detail due to many technical obstacles (15, 16).

In this paper, a new in vitro tissue culture system is introduced for the maintenance, growth, differentiation, and calcification of cells isolated from bone and periosteum. The cultures are shown to contain functioning bone cells. Histological and biochemical data are presented confirming the formation of calcified bone tissue in the cultures. Morphological features of young and old bone tissue in culture are described and compared to the fine structure of bone in vivo. A preliminary note on this work was published elsewhere (17).

MATERIALS AND METHODS

Cells and Cell Cultures

Bone cells were obtained from three sources. (a) Calvaria, comprising the frontal, parietal, and occipital bones, were removed from CR rat embryos (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), 16–20 days after gestation, washed well in phosphate-buffered saline (PBS) and separated into periosteum and bone proper. Each tissue was treated separately. The separated tissues were minced well individually to give very small pieces. These were incubated for 2 h at 37°C in a 1:1 mixture of trypsin solution 0.25% (1:300, ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio). 0.137 M NaCl, 5.4 × 10⁻³ M KCl, 4.17 × 10⁻³ M NaHCO₃, 5.5 × 10⁻³ M glucose, and a solution of disodium versenate (EDTA) (0.02%), 0.137 M NaCl, 2.7 × 10⁻³ M MCl, 8.1 × 10⁻³ M NaH₂PO₄, and 1.47 × 10⁻³ M KH₂PO₄. This solution was replaced every 30 min, and cells were harvested by slow centrifugation (100 g) in a serological centrifuge (model 2070, Falcon Plastics, Div. Becton, Dickinson and Co., Oxnard, Calif.). The cells were counted by means of a hemocytometer; there were approximately 2 × 10⁵ cells per calvarium and 10⁶ cells per periosteum. Secondary rat embryo cell cultures (18) served as a fibroblast control for the calvaria and periosteum cell cultures.

(b) The periosteum was circumferentially peeled from 3-w-old CR male rats, according to a method previously described (19). 24 h after the operational trauma, at a time when a veritable explosion of cell division takes place, the traumatized tissue was removed, minced well, and incubated in the trypsin-EDTA mixture as described under (a). Sham-operated animals were used as controls.

The cells obtained by both techniques were suspended in BGF medium (Gibco formula, [Grand Island Biological Co., Grant Island, N. Y.] “pepped up” by Fitton-Jackson’s modification), supplemented with 10% fetal calf serum (FCS) (Gibco), counted, and inoculated in 60 × 15-mm plastic petri dishes (Falcon Plastics, Division of B-D Laboratories, Los Angeles, Calif.). Seeding was at 2 × 10⁴ cells per plate. The plates were incubated up to 8 wk at 37°C in moist 90% air and 10% CO₂. The medium was renewed every 3–4 days.

Histochemical Methods

STAINING: Routine staining procedure was according to Giemsa after methanol fixation.

ALKALINE PHOSPHATASE: The Gomori Cobalt method was used, which produced a black stain. The p-nitrophenyl phosphate-agar method (20) for the detection of alkaline phosphatase-containing cells in tissue culture was also employed.

CALCIUM PHOSPHATE: Von Kossa’s silver method (21), which produced a black stain, was used.

ELECTRON MICROSCOPY: Pieces of tissue were removed from the petri dishes with a scalpel and fixed with 3% glutaraldehyde (Schuchardt, Munich) for 60 min. The fixative was washed off with cacodylate buffer (0.15 M, pH 7.4), and the samples were stained with a 1% solution of osmium tetroxide for 60 min, dehydrated in alcohol and embedded in Epon. The sections were counterstained with saturated uranyl acetate solution for 30 min, and examined in a JEOL Jem-T7 electron microscope.

PHOTOGRAPHS: Phase-contrast and bright-field micrographs were taken with a Zeiss phase-contrast inverted microscope, (Carl Zeiss, Inc., New York) on Kodak Pan-X film (Eastman Kodak Co., Rochester, N. Y.).

Calcitonin Effect

The plates used for the calcitonin (CT) experiments (5 × 10⁶ cells per plate) were washed three times with Earle’s buffer solution (15 ml total). 2 ml of Earle’s solution containing 5 mg/µl Ca²⁺ and 10¹⁷ cpm ⁴⁶CaCl₂ (Radiochemical Centre, Amersham, Great Britain), 120 µCi/µg Ca²⁺ were added, and the cultures were incubated for 60 min at room temperature to reach a plateau in ⁴⁶Ca²⁺ uptake. Sterile lyophilized porcine calcitonin, 10.23 metabolic clearance rate units/mg
Armour Pharmaceutical Co., Phoenix, Ariz.) was added to the culture, 0.1 M metabolic clearance rate units per plate. Samples were taken from the medium for counting at 5-min intervals for up to 60 min after CT addition. Then the medium was removed and the culture was washed three times with Earle's buffer solution (15 ml total). The culture was subsequently dissolved in 1 ml 0.1 N NaOH and counted in a liquid scintillation spectrometer (Packard Tricarb, Packard Instrument Co., Inc., Downers Grove, Ill.), in a dioxane-based scintillation liquid. The protein concentration was determined by the method of Lowry et al. (22).

RESULTS
Use of the different sources for isolated cells, as described in Materials and Methods (lifted periosteum, embryonic periosteum, and embryonic calvaria proper), resulted in cell cultures with closely reproducible morphological and histochemical characteristics. The adherence of the cells to the surface of the dishes occurred during the first 24 h after inoculation and was followed by cell proliferation. The general growth curves of the different cultures are shown in Fig. 1. The cells originating from lifted periosteum and from embryonic calvaria reached confluency at a cell density of approximately 0.9 \times 10^6 cells per plate (3.2 \times 10^4 and 3.0 \times 10^4 cells per cm², respectively), and their doubling-times were 2 and 4 days, respectively. The cells originating from embryonic periosteum reached confluency at a cell density of approximately 0.5 \times 10^6 cells per plate (1.8 \times 10^4 cells per cm²). Normal rat embryonic fibroblasts, however, reached confluency at the higher cell density of 4 \times 10^6 cells per plate (1.4 \times 10^5 cells per cm²) and their doubling-time was 24 h.

Figs. 2 and 3 show cultured bone cells in different stages of growth. The first stage (Fig. 2) shows a layer of undifferentiated osteoprogenitor cells showing a fibroblast-like morphology. Small clusters of osteoblast-like cells migrated away from the layer and developed further to form a network of cell processes typical of osteocytes. The clusters of osteocytes differ from the lower embryonic osteoprogenitor cell layer by their bright halos in the phase-contrast field.

The long and branched processes extending away from the cell body remained in contact with neighboring cells throughout the life of the culture, thus forming an interlaced network of cell processes (Fig. 2). During the second and third weeks in culture, the extracellular matrix, probably containing bone collagen secreted by the cells, covered most of the cells and it became impossible to differentiate clearly the cell boundaries from the matrix (Fig. 3). The osteoid tissue, formed at this stage, was the result of an advancing process of calcification that took place within the collagenous matrix. Bundles of ossified material can be clearly observed and resemble the in vivo structure of bone.

Histochemical Staining
Bone cell cultures stained with silver nitrate for calcium phosphate are shown in Figs. 4 and 5. Fig. 4 shows a 4-wk old culture. The calcified part is clearly seen in the middle of the plate. A small piece of calvarium was inserted into the culture and was used as a positive control. The calcium phosphate deposits in the culture occurred in two forms: as a diffuse layer, and as a large number of isolated black dense spots. At the end of the culturing period (after 8 wk) most of the bone tissue culture appeared black after staining (Fig. 5).
Figure 2  Bone cells 20 days in culture. Most cells show osteoblast-like morphology forming a typical network of cell processes. Phase-contrast field. × 250.

Figure 3  Bone cells 30 days in culture. The borders between the cells are hardly seen; most of the intracellular spaces contain the external matrix, probably composed mainly of bone collagen. Phase-contrast field. × 250.

Figure 4  Von Kossa staining for calcium phosphate of a 4-wk old bone cells culture. The areas in the culture stained black show positive reaction for the staining and presence of calcium phosphate. Note the positive reaction of the small piece of calvaria bone inserted in the left. Direct-light field. × 125.

Figure 5  Von Kossa staining for calcium phosphate of an 8-wk old bone cells culture. In this stage all the culture is stained by this staining. Direct-light field. × 125.
Osteoblasts are known to contain an elevated amount of alkaline phosphatase (23). Most of the enzymatic activity is associated with the cell membranes (23, 24). For this reason histochemical staining for alkaline phosphatase was also performed. Alkaline phosphatase activity was found to be located at the surface of the bone cells, as shown by their black envelope. A different staining procedure for alkaline phosphatase, based on the agar method of Maio and coworkers (20), was also used. In the bone cell cultures the agar was stained by the yellow derivative of p-nitrophenol, establishing the presence of alkaline phosphatase activity in the bone cell culture. Only negligible staining occurred in rat fibroblast culture.

The Effect of Calcitonin

Calcium uptake by cultured bone cells, as measured by $^{45}\text{Ca}$ incorporation, was found to be four times as high as by skin fibroblasts under the same conditions. CT added to bone cell cultures caused a significant rise in $^{45}\text{Ca}$ uptake by these cells. The hormone, however, had no effect on the cultured rat fibroblasts (Table I).

Electron Microscopy

The fine structure studies of the culture were directed in particular to the process of mineralization. Results are presented concerning the ultrastructure of the cultured bone cells before and after the occurrence of calcification.

Uncalciﬁed Young Culture: During the differentiation period, osteoblasts surrounded by fine collagen fibers are characteristic of bone tissue formation in culture. A striking feature of a young culture (1–3 wk) is the occurrence of osteoblasts in clusters. In the 3-wk culture (Figs. 6 and 7) numerous active osteoblasts are visible. In the cells large indented nuclei with well-deﬁned membranes are seen. Fig. 7 shows a typical culture of such young bone cells. The cells within a cluster show various structural features at their sites of contact. The cell membrane surfaces are either closely apposed or separated from each other by extracellular matrix spaces of variable width which contain mainly short and longer collagen ﬁbrils. The cytoplasm is rich in ribosomes both free, and bound to a well-developed rough endoplasmic reticulum in large mitochondria and in membranous vesicular structures of the Golgi complex (Fig. 7). The latter structures indicate an active secretory function. The cytoplasm sometimes contained numerous myelin-like figures also termed lamellar body membranes (25, 26). These laminated membranous cytoplasmic bodies occur in the near vicinity of the mitochondria (Figs. 6–8 and 10). The lamellae composed of electron-dense, layered membranes are arranged either concentrically, as shown in cross sections (Figs. 7 and 8), or in parallel, as shown in longitudinal sections (Figs. 8 and 10). These lamellar structures are known to exist in a variety of cells such as lung great alveolar cells (25) or neuronal cells (26). Their appearance in bone cells, however, has not been previously reported.

The extracellular matrix of the young culture contains unmineralized thin collagen fibrils varying in size and diameter (Figs. 6, 7, 9, and 10). Some adjacent fibrils are registered to accurately that their banding pattern appears continuous (Fig. 9).

Noncalcified matrix contains vesicular structures and amorphous-looking materials which may be composed of mucoproteins or glycoproteins (Fig. 10), and which may play an important role in the calcification process.

Calcifying Culture: Mineralized matrix appears at later stages (8 wk). The deposition of crystals of calcium phosphate in the extracellular matrix of isolated cultured bone cells strongly supports our contention that it is possible to obtain bone formation in this system. Although calcification of the matrix usually occurs in the near vicinity of the collagen fibrils (Figs. 11 and 12), no relationship between the position of the needle-shaped apatite crystals and the collagen banding pattern was visible. Careful inspection of Fig. 12 reveals the presence of vesicle-like structures randomly dispersed in the collagen matrix containing some apatite crystals. Further calcification of the matrix may occur by continued mineral deposition in these structures. Eventually, massive calcification occurs also between these structures, giving rise to large mineralized areas within the matrix. These subsequent stages can be observed.

<table>
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<th>CT (mU MRC/plate)</th>
<th>Bone cells $^{45}\text{Ca}$ uptake (cpm/mg protein) $\times 10^{-3}$</th>
<th>Skin fibroblasts $^{45}\text{Ca}$ uptake (cpm/mg protein) $\times 10^{-3}$</th>
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<tr>
<td>0.1</td>
<td>4.8 ± 0.3</td>
<td>1.2 ± 0.3</td>
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<td>7.4 ± 0.3</td>
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in Fig. 13. The fully mineralized matrix appears opaque and electron dense, and shows a marked osmiophilic border line separating the well-calci-fied matrix from the partially calcified one (Fig. 13). The relationship between the cells and the calcified matrix is also shown in Fig. 13. Interestingly, a gradient of calcification exists, the less calcified matrix lying closest to the cell surface.

Three stages of the mineralization process in this culture are therefore demonstrated. The first stage consists of the appearance of individual crystals close to the collagen fibrils. Densely mineralized islands are formed during the second stage. Organization and concentration of these loci form highly mineralized bone matrix.

**Discussion**

Under normal conditions, bone of adult animals shows only slight osteogenic activity (27). In order to study osteogenesis, conditions were used in which osteogenic activity is either originally higher or is stimulated. The source of bone cells was embryonal calvaria rudiment, its periosteum, or periosteum stimulated by surgical lifting. Cultures of cells obtained in these different ways showed some of the main characteristics of bone cells in vivo. Cells derived from periosteum, however, are more suitable for culturing. The number of cells that can be obtained from lifted periosteum by digestion, their doubling time, and their saturation density are relatively more favorable as compared to these values for cells from other sources. It seems therefore that periosteum could be used best as a source for cells in experimental studies concerning differentiation of bone tissue in vitro (5). Positive reactions to histochemical staining for calcium ions and for alkaline phosphatase were demonstrated in the cultures. It was shown by Nichols et al. (4) that isolated bone cells preferentially accumulated larger amounts of
FIGURE 7  Young bone cells 3 wk in culture. Cells show indented nuclei N, Golgi apparatus GA, abundant endoplasmic reticulum ER, vacuoles Vc, and myelin-like figures MP. The matrix contains collagen fibrils Co. Glutaraldehyde-fixed, osmium-postfixed, 0.5 µm section. X 30,000.
Figure 8  The myelin-like figure MF found in bone cells cultured for 3 wk. Note the lamellar concentric arrangement in the cross section and the parallel arrangement in the longitudinal section. Endoplasmic reticulum ER, mitochondria M. Glutaraldehyde-fixed, osmium-postfixed, 0.5 µm section. X 50,000.

Figure 9  Banding pattern of the collagen fibrils Co in the extracellular matrix of bone cell culture, for 3 wk. Endoplasmic reticulum ER. Glutaraldehyde-fixed, osmium-postfixed, 0.5 µm section. X 43,500.
calcium than do other cells in the organism. Moreover, bone cells have an increased calcium uptake when incubated in the presence of calcitonin (28). Our technique, by which isolated (28) and cultured bone cells react similarly and exhibit an increase in calcium uptake in response to calcitonin, may serve as a significant method for the identification of metabolically active bone cells.

This method permits observation in culture dishes of the different stages of calcification of the extracellular matrix, and makes it possible to compare this process to bone formation in vivo.

The study of bone tissue formation in cell cultures has several advantages. a Bone specimens prepared for electron microscopy are often subjected to prolonged decalcification (4), which is not necessary for the cultures. b The vital properties of the cells are more readily conserved in tissue culture than in whole bone tissue, since the fixatives are more accessible to the thin culture specimens. c Bone tissue formation can easily be followed step by step.

The overall picture of cultured bone cells and the matrix is similar to that of the bone tissue described in in vivo studies (14–16). In the early stages, short collagen fibrils are observed adjacent to, or merging with, the cell surface of the osteoblasts. Similarly, such structures were found also in embryonic fowl osteoblast cultures (29). Well-developed rough endoplasmic reticulum, numerous mitochondria, a vesicle-rich Golgi apparatus (30), and both microtubules and microfilaments in the inner parts of the cell surface indicate that these cells actively synthesize matrix components.

Recent biochemical findings (31–33) suggest that transformation of procollagen into collagen occurs at the cell membrane, and that the secreted collagen aggregates into fibrils immediately after secretion through the cell membrane. The morphological appearance of cultured bone cells and its matrix support this suggestion.

In the early stages of bone tissue formation in our culture, fine mineral crystallites are present embedded in an electron-dense, granular material.
Figure 11  Mineralized collagen fibrils from a six-wk old bone cell culture. Glutaraldehyde-fixed, osmium-postfixed 0.5 µm section. X 27,800.

Figure 12  Extracellular matrix of bone cells cultured for 8 wk. Partially mineralized collagen fibrils, Co, and vesicles, v. Glutaraldehyde-fixed, osmium-postfixed, 0.5 µm section. X 30,000.
FIGURE 13  The mineralization gradient in the bone cells cultured for 8 wk. Nucleus N, unmineralized collagen fibrils Co, initial mineralization IM, partial mineralization PM, full mineralization FM, microfilaments F. Glutaraldehyde fixed, osmium-postfixed, 0.5 µm section. X 25,000.
coating small groups of collagen fibrils. This observation is in agreement with the results of previous studies showing that the collagenous matrix is the site for deposition of calcium phosphate crystals. This has been demonstrated in cultured embryonic fowl bone (34), in rachitic osteoid tissue healing in vivo (35), in calcifying turkey leg tendons (36), in reconstituted collagen fibrils calcifying in vitro (37, 38), and in demineralized bone matrix calcifying in a metastable buffer (39).

While apatite crystals appear to be arranged along the collagen fibers, a close morphological relationship between the collagen banding-pattern and the position and orientation of the crystals was not apparent. Such findings were also reported elsewhere (16).

Further calcification leads to patches of mineral deposition with no definite relation to collagen fibers. These patches seem to be connected with vesicle-like structures, which may be similar to the noncollagenous bone nodules described elsewhere (40, 41).

During the last stage of bone development in culture, fully calcified areas appear. These areas are usually bordered by a distinct osmiophilic line. A gradient of calcification exists, the fully calcified matrix being furthest removed from the bone cells. There is no doubt, however, that the bone cells exert some control over the mineralization of the matrix surrounding them. This has been suggested to be the case during the initiation of the mineralization process (42). At this stage it is not known whether the bone cells also actively participate in the massive calcification, or this process occurs by a physicochemical mechanism of crystal nucleation and growth.

Chemical analysis of the collagen produced by bone cells in culture showed that the chemical composition of this collagen with respect to aldehydes and cross-links is similar to that of collagen produced by bone tissues in vivo (43).

Our system for culturing bone cells appears to be a successful attempt to grow bone tissue in vitro starting from isolated bone cells. Formation of bone tissue by these cells is indicated by the following findings: (a) the cells show osteoblastic morphology in light and electron microscopy; (b) the collagen formed is similar to bone collagen; (c) Specific mineralization occurs in the culture, and the mineral stains by the von Kossa method; (d) The cells react to calcitonin hormone and take up more calcium ions in the presence of the hormone.

The introduction of this tissue culture system for investigation of the differentiation, growth, and function of bone cells enables us to study the role of the cells and their constituents in the different stages of bone formation, and the influence of hormones and other factors in this process.

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