Bone Cells: A Serum-Free Medium Supports Proliferation in Primary Culture

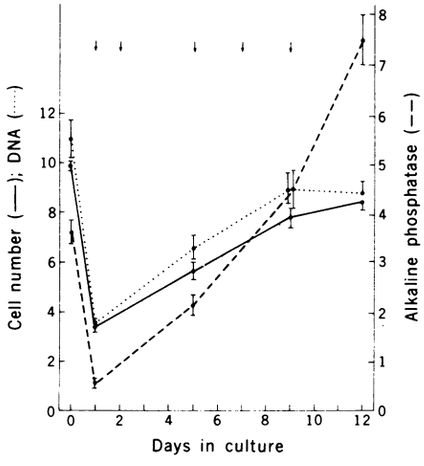
Abstract. *Bone cells isolated from embryonic rat calvaria increase in number two- to threefold when cultured at high, but not at low, population densities in a serum-free medium that contains albumin. Cultured cells respond to parathyroid hormone and exhibit a marked rise in alkaline phosphatase activity during proliferation, which suggests the progressive differentiation or preferential growth of osteoblast-like cells.*

Studies in which isolated bone cells in primary culture are used have advanced our understanding of the actions of major bone-seeking hormones (*1*). Cultured cells are sensitive to near physiological concentrations of several hormones and retain the capacity to perform differentiated functions (*2, 3*). A strict requirement for serum to support the growth of bone cells in vitro, however, has impeded further clarification of the regulation of cell proliferation and differentiation, since serum contains defined and undefined growth-modifying substances (*4*). Attempts to grow nonskeletal cells in the absence of serum have met with but limited success. Additions of specific nutrients, hormones, or polypeptides reduce, but do not eliminate, serum requirements for growth of certain cell types in primary culture (*5*). Although several established cell lines have been found to grow in serum-free medium, they are less suitable than primary cultures for studies of differentiated functions (*5*). We report the development of a serum-free medium that supports the proliferation of isolated bone cells in primary culture.

Bone cells were isolated from the frontal and parietal bones of rat fetuses 19 to 21 days old, as previously described (*1*). Cartilage and periosteal connective tissue were removed by sharp dissection prior to enzymatic digestion with crude collagenase (*1*). Cultures were seeded at an initial density of 10^6 cells (10^5 cell/cm^2) per Falcon petri dish (diameter, 35 mm) and were maintained in a humid atmosphere of 2 percent CO_2 and 98 percent air. Cells were grown in the Fitton-Jackson BGJ_b medium (*6*), previously used to study bone cell metabolism (*7*), and modified so that its ionic composition resembled that of the bone fluid as described by Neuman and Ramp (*8*) (Table 1). The sodium and potassium concentrations were 125 m*M* and 25 m*M*, respectively. This medium (2.0 ml) was replaced three times a week. Cell number (after trypsinization), alkaline phosphatase activity, and DNA content were estimated on days 1, 5, 9, and 12 of culture (Fig. 1). Replicate cell cultures were exposed to [^{3}H]thymidine (1.0 μc per 2.0 ml of culture medium) (*9*) for 1 hour, and the radioactivity in the acid-insoluble fraction (*10*) was estimated with a Packard Tri-Carb liquid-scintillation spectrophotometer.

Cells began to attach to the plastic surface within 5 minutes of seeding. Although 35 percent of the inoculated cells remained firmly attached after the first 24 hours, failure to attach was not related to cell death, since 75 percent of the unattached cells were viable as judged by trypan blue exclusion. In view of the well-known limitations of dye exclusion methods in assessing cell integrity, the unattached cells may have been damaged sufficiently to prevent attachment. Alternatively, the attached cells may represent a different type of cell than the remainder of the aggregate cell harvest. Microscopic inspection of the culture plates suggested cell proliferation by day 5. This impression was confirmed by the presence of statistically significant increases in the cell number and total DNA that continued through day 9 (Fig. 1). The incorporation of [^{3}H]thymidine rose progressively to a maximum of 42,160 ± 2,232 disintegrations per minute (dpm) per culture (mean ± standard error of the mean) on day 7, then gradually fell to 17,237 ± 1,281 dpm per culture on day 12, by which time the cultures were nearly confluent. The conditions used in these experiments consistently supported bone cell proliferation. Figure 1 presents data accumulated from six separate experiments. Bone cells cultured at lower initial population densities (5×10^4 cells per square centimeter) failed to proliferate. Instead there was gradual cell attrition over a 12-day period.

Three lines of evidence suggest the



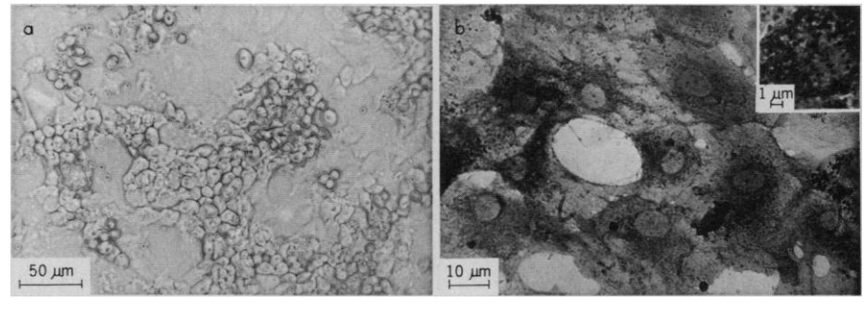


Fig. 1 (left). Growth of isolated subperiosteal bone cells seeded at an initial population density of 10^5 cell/cm^2. Each point represents the mean and standard error of data pooled from six separate experiments. Day 0 represents the initial inoculation; all other points represent firmly attached cells removed from the plates after trypsinization. Solid line is cell number × 10^5 per culture; dashed line, alkaline phosphatase activity [(micromoles of *p*-nitrophenol per hour) × 10^3]; dotted line, DNA in micrograms per culture. Arrows represent the days on which the medium was replaced. Following enzymatic digestion of the calvaria, the dispersed cells were rinsed four times with isolation medium, allowed to stand at room temperature for 5 minutes in isotonic ammonium chloride to lyse red blood cells (*22*), and then washed and suspended in isolation medium for hemocytometer counting. Alkaline phosphatase activity was measured by the method of Koyama and Ono (*23*) at a *p*H of 10.3. DNA was determined by the indole method of Hubbard *et al.* (*24*), in which hydrolysis for 20 minutes at 90°C and a volume of 1.0 ml was used, or by the diphenylamine method of Leyra and Kelley (*25*). Both methods gave identical results when corrected for recovery of internal standards. Fig. 2 (right). Morphological characteristics of bone cells cultured for 12 days in bone fluid medium. (a) Phase-contrast photomicrograph demonstrating dense aggregates of cells in a near confluent monolayer. (b) Cytoplasmic and nuclear alkaline phosphatase activity. Cultures were fixed at 4°C (30 seconds) with 10 percent formalin in methanol and stained by the method of Ackerman (*11*). Inset represents the region where cells form dense aggregates. Cells devoid of alkaline phosphatase activity are not visible.

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preferential growth or differentiation (or both) of osteoblast-like cells in this culture system. (i) A marked rise in alkaline phosphatase activity per cell accompanied cell growth (Fig. 1). (ii) The number of cells that contained prominent cytoplasmic alkaline phosphatase activity increased from less than 1 percent on day 1 to more than 25 percent on day 12 of culture (Fig. 2). (iii) Cells cultured for 9 to 12 days responded to parathyroid hormone [synthetic bovine parathyroid hormone, amino acids 1-34 (*11, 12*), 100 ng/ml for 5.0 minutes] with a tenfold increase in 3′,5′-adenosine monophosphate (cyclic AMP) when treated as described (*2*) (data not shown). There is every reason to believe that osteoblasts contain copious amounts of cytoplasmic alkaline phosphatase (*13*). Luben *et al.* (*14*) have provisionally characterized as osteoblasts those bone cells that are rich in alkaline phosphatase and respond to parathyroid hormone with large increases in cyclic AMP. The observed increase in alkaline phosphatase activity might reflect recovery from damage to surface enzyme caused by exposure of the bone cell to collagenase during isolation, rather than de novo differentiation. In favor of the latter interpretation, however, is the finding of Miedema (*15*) that the loss of alkaline phosphatase activity which accompanied collagenase treatment of an established cell line was not reversible. Definitive identification of the surviving cells as osteoblasts will require the demonstration that they are capable of producing bone.

Miedema (*15*) has demonstrated a similar density-dependent increase in alkaline phosphatase activity in cultured human HEp2 and WISH cells, as have Binderman *et al.* (*7*) in an isolated bone cell system. In each case, the incubation medium was supplemented with fetal calf serum. Yamane *et al.* (*16*) have initiated primary cultures of ascites tumor cells in a defined medium supplemented with 1 percent fat-containing serum albumin. In addition to providing essential fats, albumin may have served to trap toxic substances (*17*). Fat-poor albumin, such as that used in our system, was not found to support the growth of the ascites cells (*16*).

To determine whether the unusual ionic composition of the incubation medium used in our experiments (125 m*M* sodium, 25 m*M* potassium) influenced cell growth and apparent differentiation, we examined the effect of a medium containing more physiologic concentrations of sodium (145 m*M*) and potassium (5 m*M*). Direct comparison of these media revealed no differences in plating efficiency or in the rate of cell proliferation. Cells cultured in the standard medium, however, exhibited only 50 percent of the maximum alkaline phosphatase activity and were only 50 percent as responsive to parathyroid hormone (see above) as those grown in the low sodium–high potassium medium (data not shown). No proliferation was observed in medium containing 155 m*M* sodium and 25 m*M* potassium (mean osmolality, 385 milliosmoles). Hence proliferation may be sensitive to osmolality, and apparent differentiation to the concentrations of sodium and potassium.

Isolated periosteal cells, removed from the superior and inferior periosteal surfaces by sharp dissection prior to collagenase digestion (*18*), failed to survive in the serum-free medium even when

Table 1. Comparison of Fitton-Jackson BGJ_b with bone fluid medium. Fitton-Jackson modified BGJ_b medium (Gibco) has a mean osmolality of 390 milliosmoles. To prepare the bone fluid medium, the salts and organic components are dissolved in 6 to 7 dl of glass-distilled deionized water. One deciliter of commercially prepared BGJ_b (Fitton-Jackson modified) amino acids and vitamins (10 ×) without ascorbic acid and glutamine is added (Gibco, quotation No. 8202) followed by 1000 mg of Pentex albumin (Miles Laboratories). The *p*H is adjusted to 7.45 at room temperature with 0.4*M* NaOH and the volume adjusted to 1 liter; $NaHCO_3$ is added prior to membrane sterilization. The final *p*H is 7.50 to 7.55, which is optimum for cell attachment and collagen biosynthesis (*21*). The mean osmolality of the bone fluid medium is 350 milliosmoles. The isolation medium is prepared in a similar manner but has a final *p*H of 7.00.

Component	Concentration	
	Fitton-Jackson BGJ_b (mg/liter) (mole/liter)	Bone fluid medium (mole/liter) (mg/liter)
$CaCl_2 \cdot 2H_2O$		73.52 (5.0×10^{-4})
Calcium lactate	555.00 (1.8×10^{-3})	
$MgSO_4 \cdot 7H_2O$	200.00 (8.1×10^{-4})	98.60 (4.0×10^{-4})
K_2HPO_4		261.28 (1.5×10^{-3})
KH_2PO_4	160.00 (1.2×10^{-3})	
$NaH_2PO_4 \cdot H_2O$	90.00 (6.5×10^{-4})	41.40 (3.0×10^{-4})
NaCl	5,300.00 (9.1×10^{-2})	5,844.00 (1.0×10^{-1})*
KCl	400.00 (5.4×10^{-3})	1,640.26 (2.2×10^{-2})
$NaHCO_3$	3,500.00 (4.2×10^{-2})	1,260.60 (1.5×10^{-2})
$FeSO_4 \cdot 7H_2O$		0.83 (3.0×10^{-6})
Hepes†		4,766.00 (2.0×10^{-2})
D-Glucose	10,000.00 (5.5×10^{-2})	2,000.00 (1.1×10^{-2})
Sodium acetate ($3H_2O$)	50.00 (6.0×10^{-4})‡	54.43 (4.0×10^{-4})
α-Ketoglutaric acid		29.22 (2.0×10^{-4})
Phenol red	20.00 (5.6×10^{-5})	5.00 (1.4×10^{-4})
L-Alanine	250.00 (2.8×10^{-3})	250.00 (2.8×10^{-3})
L-Arginine	175.00 (1.0×10^{-3})	175.00 (1.0×10^{-3})
L-Aspartic acid	150.00 (1.1×10^{-3})	150.00 (1.1×10^{-3})
L-Cysteine HCl · H_2O	90.00 (5.1×10^{-4})	90.00 (5.1×10^{-4})
L-Glutamine	200.00 (1.4×10^{-3})	292.3 (2.0×10^{-3})
Glycine	800.00 (1.1×10^{-2})	800.00 (1.1×10^{-2})
L-Histidine	150.00 (9.6×10^{-4})	150.00 (9.6×10^{-4})
L-Isoleucine	30.00 (2.3×10^{-4})	30.00 (2.3×10^{-4})
L-Leucine	50.00 (3.8×10^{-4})	50.00 (3.8×10^{-4})
L-Lysine	240.00 (1.3×10^{-3})	240.00 (1.3×10^{-3})
L-Methionine	50.00 (3.4×10^{-4})	50.00 (3.4×10^{-4})
L-Phenylalanine	50.00 (3.0×10^{-4})	50.00 (3.0×10^{-4})
L-Proline	400.00 (3.5×10^{-3})	400.00 (3.5×10^{-3})
L-Serine	200.00 (1.9×10^{-3})	200.00 (1.9×10^{-3})
L-Threonine	75.00 (6.3×10^{-4})	75.00 (6.3×10^{-4})
L-Tryptophan	40.00 (2.0×10^{-4})	40.00 (2.0×10^{-4})
L-Tyrosine	40.00 (2.2×10^{-4})	40.00 (2.2×10^{-4})
DL-Valine	65.00 (5.6×10^{-4})	65.00 (5.6×10^{-4})
α-Tocopherol phosphate	1.00 (2.3×10^{-6})	1.00 (2.3×10^{-6})
Ascorbic acid	50.00 (2.8×10^{-4})	50.00 (2.8×10^{-4})
Biotin	0.20 (8.2×10^{-7})	0.20 (8.2×10^{-7})
Calcium pantothenate	0.20 (4.2×10^{-7})	0.20 (4.2×10^{-7})
Choline chloride	50.00 (3.6×10^{-4})	50.00 (3.6×10^{-4})
Folic acid	0.20 (4.5×10^{-7})	0.20 (4.5×10^{-7})
i-Inositol	0.20 (1.1×10^{-6})	0.20 (1.1×10^{-6})
Nicotinamide	20.00 (1.6×10^{-4})	20.00 (1.6×10^{-4})
p-Amino benzoic acid	2.00 (1.6×10^{-5})	2.00 (1.6×10^{-5})
Pyridoxal phosphate	0.20 (8.1×10^{-7})	0.20 (8.1×10^{-7})
Riboflavin	0.20 (5.3×10^{-7})	0.20 (5.3×10^{-7})
Thiamine-HCl	4.00 (1.2×10^{-5})	4.00 (1.2×10^{-5})
Vitamin B_{12}	0.04 (2.9×10^{-8})	0.04 (2.9×10^{-8})

*The amount of NaCl added depends upon the NaCl content of the commercially obtained amino acid–vitamin mixture. The final ion concentrations are similar to the bone fluid described by Neuman and Ramp: Na^+, 125 m*M*; K^+, 25 m*M*; Ca^{2+}, 0.5 m*M*; Mg^{2+}, 0.4 m*M*; Cl^-, 130 m*M*; and P_i, 1.8 m*M* (*8*). †*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid. ‡Anhydrous sodium acetate.

seeded at high initial population densities (10^5 cell/cm^2). Seventeen percent of the cells attached, but only 5 percent remained by day 5. The alkaline phosphatase activity was low (28.3 ± 5.3 μmole per hour per culture) in comparison with that of cell cultures prepared from subperiosteal bone. Fibroblasts obtained by collagenase digestion of fetal rat skin died over the initial 2- to 3-day period in culture.

Growth and apparent differentiation at a high, but not at a low, initial population density may reflect the continuing action of tissue or serum growth factors, or both, that adhered to the cells during preparation. Alternate explanations include (i) the elaboration by cultured cells of their own growth factors or essential nutrients that are absent from the medium (*19*) and (ii) inactivation by the larger cell mass of toxic substances in the medium (*20*). The latter possibility would explain not only the failure of cells to proliferate when cultured at low density but also the low plating efficiency. Although there was appreciable cell death during the first 24 hours in culture, stimulation of proliferation cannot be attributed easily to growth-promoting factors released into the incubation medium by the dying cells. The incubation medium was replaced completely at 24 hours (Fig. 1), whereas proliferation was evident long afterward. Moreover, fibroblasts and periosteal cells did not proliferate, despite greater initial cell attrition. The system described herein should permit clarification of the mechanism of density-dependent proliferation and an examination of the effects of growth factors on the proliferation and differentiation of bone cells in vitro in the absence of complex and ill-defined serum additives.

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Cyclopropanecarboxylic Acid: Chain Elongation to ω-Cyclopropyl Fatty Acids by Mammals and Plants

Abstract. *Rats dosed orally with* [carboxyl-^{14}C]*cyclopropanecarboxylic acid (or its hexadecyl ester) retain radioactivity in tissue as novel triacylglycerols. The most abundant* ^{14}*C-labeled metabolites were identified by gas-liquid chromatography–mass spectrometry as 13-cyclopropyltridecanoic and 15-cyclopropylpentadecanoic acids. Similar ω-cyclopropyl fatty acids are produced by beagle dogs and a lactating cow, as well as by apple and orange trees.*

Certain esters of cyclopropanecarboxylic acid (CPCA) are selectively toxic to phytophagous Acarina (spider mites) without significant insecticidal activity (*1, 2*). We have studied the metabolic fate of the hexadecyl ester of CPCA (cycloprate), which is currently under commercial development as a miticide. We have identified unusual metabolites arising from the apparent entry of CPCA into pathways of fatty acid anabolism, with net additions of acetate to give a homologous series of ω-cyclopropyl fatty acids.

Duncombe and Aising (*3*) studied the metabolism of ^{14}C-labeled CPCA in rat tissue in vitro and postulated the formation of unsaturated ω-cyclopropyl fatty acids, but were unable to identify these products. Linscott *et al.* (*4, 5*) have shown that plants can elongate the carbon chains of 2,4-dichlorophenoxyalkanoic [for example, -acetic (2,4-D) and -butyric] acids by insertion of pairs of methylene groups. The major metabolites of these acids were identified as resulting from addition of one to three acetate units (that is, two to six CH_2 groups), whereas we find addition of up to eight acetates to CPCA. We know of

Table 1. Quantitative abundance of ω-cyclopropyl fatty acids, as a percent of the administered dose of [^{14}C]cycloprate, in portions of various organisms that were analyzed (*6*).

ω-Cyclopropyl fatty acids*	Rat carcass	Cow milk	Dog carcass	Apple fruit	Orange fruit
8(5cPr) : 0		0.2			
10(7cPr) : 0		0.3			
12(9cPr) : 0		0.3			
14(11cPr) : 0	1.0	0.7	0.4		
16(13cPr) : 0	9.9	1.6	6.5		
18(15cPr) : 0	2.5	0.2	2.7	4.5	7.6
18(15cPr) : 1				1.9	3.7
20(17cPr) : 1					0.3

*Structural abbreviations are explained in the legend to Fig. 1.

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