

Bone Formation and Calcification by Isolated Osteoblastlike Cells

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ABSTRACT Two cell populations were isolated from calvaria of chick embryos: PF cells were liberated by collagenase treatment from the periosteum, OB cells from the periosteum-free calvarium. Both populations were cultured in plastic culture dishes. After 6 d of culture, monolayers of each cell type either were scraped off the culture dishes, transplanted on the chorio-allantoic membrane of 7-d-old quail eggs, and cultured there for 6 d, or were used for biochemical experiments.

OB transplants proved capable of producing calcified bone matrix, whereas PF transplants formed only fibrous tissue. Biochemically, OB cells showed high cAMP production in the presence of parathyroid hormone (PTH), whereas cAMP production was not stimulated in PF cultures. Lactate production was stimulated by PTH in both populations although somewhat differently. Citrate decarboxylation was high in OB cells and was inhibited by PTH but was low in PF cells, where it was stimulated by the same hormone. The differences in hormonal response between the two cell types made it possible to conclude that PF cultures are relatively free of OB cells. The PF contamination in OB cultures was more difficult to assess.

The experiments described in this report show that the OB population contains osteoblasts or osteoblastlike cells which are, under favorable circumstances, capable of bone formation.

Understanding of the metabolism of bone tissue and its hormonal regulation has been considerably increased by the study of different types of bone cell after they have been separated from each other. Several procedures have been used to obtain these different types of bone cell (13, 17, 20, 21, 23). Generally, the isolated cells are cultured as a monolayer for a number of days and then used for biochemical studies. This procedure has the serious drawback that, after isolation and culture, the cells have lost many of the specific features permitting their identification. For example, cells isolated from the osteoblastic layers of embryonic calvaria and thought to be osteoblasts lose their cuboidal shape when grown in monolayer culture. Furthermore, these cells are capable of collagen production *in vitro* but formation and calcification of bone matrix are rarely obtained. Recently, a number of investigators have reported calcification to occur in their bone cell cultures (2, 3, 18, 19, 27). However, although these studies show matrix formation and calcification in bone cell cultures, they do not provide definite proof of the ability of bone cells in monolayer culture to produce calcified bone matrix; light microscope studies do not show convincingly the presence of osteoblasts and bonelike structures (2, 3, 18, 19, 27), the organic matrix may differ from

the bone matrix *in vivo* (3), and matrix formation and calcification also occur in cultures of skin fibroblasts, albeit to a lesser extent (27).

In our previous studies we have isolated various cell types (16, 17) and characterized them on the basis of histological studies with light microscopy, transmission electron microscopy, and scanning electron microscopy on the tissues from which the cells were isolated, before and after isolation (17). In addition the isolated cells were studied biochemically during monolayer culture (17). The results of these studies led us to conclude tentatively that one of the populations of isolated cells was a population of osteoblasts or osteoblastlike cells.

In this investigation further proof of the validity of this characterization was obtained, in that the population concerned was still capable of formation of bonelike structures and calcification of this material after 6 d of monolayer culture.

MATERIALS AND METHODS

Cell Isolation

To obtain the two cell populations, calvaria of 16-d-old chick embryos were dissected and the periosteum removed from both sides of the calvaria (17). One cell

population (PF) was isolated from the periosteum, the other cell population (OB) from the remaining calvaria. Cells were isolated by treating the periosteum or calvaria three times with collagenase (3 mg/ml, crude collagenase, Sigma Chemical Co.), first once for 5 min and then twice for 60 min. The first fraction (5 min), which was discarded, contained mainly erythrocytes and damaged cells. The other two fractions were combined, washed with Hanks' buffered salt solution (BSS) containing 10% cock serum, and seeded in plastic Petri dishes. The culture medium consisted of Eagle's MEM in Earle's BSS supplemented with 10% cock serum, 10% embryonic extract of 10-d-old chick embryos, 50 µg/ml vitamin C and 250 µg/ml glutamine. Cells were cultured for 6 d; the medium was changed after 3 d.

Transplantation on the Chorio-Allantoic Membrane

After 6 d of culture the cells, PF or OB, were scraped off the bottom of the culture dish, embedded in a drop of chicken plasma, and placed on the chorio-allantoic membrane (CAM) of 7-d-old quail embryos. The technique of transplantation was approximately as described by Hamburger (7) for transplantation on the chick CAM. The windows in the shell were closed with surgical tape (steri-strip skin closure tape, 3M Co., St. Paul, MN). After 6 d the transplants were removed and fixed in Bouin-Hollande solution (22) or absolute ethanol. Serial paraffin sections were cut 5-µm thick. Half of the sections of Bouin-Hollande's-fixed transplants were stained with azur-eosin or azocarmine-aniline blue (azan staining), the other half with Feulgen reagent for differentiation between chicken and host cells (10). Sections of ethanol-fixed material were stained either with azan stain or according to von Kossa (22). In a number of cases, alkaline phosphatase activity was studied in 10-µm-thick frozen unfixed sections which had been incubated in a solution containing α -naphthylphosphate and Fast Blue RR. Where alkaline phosphatase was present an insoluble naphthyl-Fast Blue RR complex was formed during this incubation.

Biochemical Determinations

Some additional (17) biochemical experiments were performed with 6-d-old monolayer cultures. Lactate was determined according to Lowry et al. (12), DNA according to Karsten et al. (9), and for cAMP a binding assay according to Lust et al. (14) was applied to cAMP extracted from the cell cultures with 90% *n*-propanol in water (8). Citrate decarboxylation was measured by incubation of the cell cultures for 2 h at 37°C in a medium containing 0.1 µCi/ml citrate-6-¹⁴C and determination of the released ¹⁴CO₂ (4).

RESULTS

OB and PF cells grew readily in plastic Petri dishes. The morphology of cultures and cells did not differ greatly between the two types. PF cells tended to be more elongated than OB cells and grew in monolayers or bilayers, whereas OB cells generally formed multilayers. Both cell types showed collagen formation in culture, the OB more so than the PF cell cultures. Bone formation did not take place in cultures of either cell type, even after 4 wk. Sometimes, matrix condensation together with some calcium phosphate (von Kossa positive) deposition could be observed in OB cultures but neither light-microscope nor electron-microscope studies showed bone formation convincingly (unpublished results).

When 6-d-old OB cultures were transplanted on the chorio-allantoic membrane of 7-d-old quail embryos and incubated for 6 d, ~80% of the transplants showed bonelike structures (Fig. 1, Table I). The matrix of these structures stained intensely blue with the azan stain. Along the structures, cuboidal cells with heavily stained, azurophil cytoplasm and lightly stained nuclei could be seen in the azur-eosin-stained sections (Figs. 2, 3). In Feulgen's-stained sections, these typically osteoblasts were found to be of chick origin (Fig. 4). The difference between chick and quail cells could be established easily because the structure of the chromatin in the nuclei of quail cells is quite different from that in the nuclei of chicken cells (Fig. 4). This makes it possible to differentiate between chick and quail cells in chimaeras of chick and quail tissues after Feulgen

staining (10). Calcification of the bonelike structures occurred in a high percentage of the total number of transplants studied with the von Kossa stain (Table I, Fig. 5).

In transplants of 6-d-old PF cultures, bone formation and calcification did not occur (Table I), even though collagen formation took place (Fig. 6). PF transplants resembled densely populated connective tissue with collagen fibrils but not bone matrix between the cells.

The ingrowth of blood vessels from the CAM into the transplant appeared to be a very important factor for survival. All successful transplants, OB and PF alike, showed numerous blood vessels (of quail origin). Absence of blood vessel ingrowth was probably one of the major reasons for the failure of many transplants. About 25% of all cell cultures transplanted were still in a good state after 6 d on the CAM. The others died prematurely even though many of the OB transplants still showed the bonelike structures laid down during the first few days of culture.

Biochemically, too, 6-d-old cultures of OB and PF differed strikingly. The formation of cAMP was stimulated by parathyroid hormone (PTH) in OB but not in PF cultures (Fig. 7). On the other hand, lactate production could be stimulated in both cell types (Fig. 8). Even so, the sensitivity to PTH appeared to differ between OB and PF cells. Citrate-6-¹⁴C decarboxylation was inhibited in OB cultures by PTH but stimulated in PF cultures (Fig. 9). Moreover, the degree of citrate decarboxylation expressed per mg DNA was about 400-fold lower in PF cultures.

As already reported (17), alkaline phosphatase activity was much higher in OB than in PF cells immediately after isolation. In both cases, however, the activity per cell decreased considerably during cell culture although it remained higher in OB than in PF cultures throughout the 6-d culture period (17). Alkaline phosphatase activity was present in the transplants of both cell types after 6 d on the chorio-allantoic membrane. Here, too, the activity was higher in the OB, again, than in the PF transplants especially in the cells lining the bonelike structures.

DISCUSSION

Osteoblasts are characterized by (a) their typical morphology, (b) the ability to form bone matrix, (c) the capacity for calcification of bone matrix, and (d) high alkaline phosphatase activity on the outer surface of the cell. A possible fifth property is the increased intracellular cAMP production in the presence of PTH. Most of the evidence for this property is, however, circumstantial. A number of investigators have found high cAMP responses to PTH in isolated bone-cell populations where the osteoblasts were expected to be (13, 17, 21, 24). A more direct indication was provided by Davidovitch et al. (5), who showed with immunohistochemical techniques that, in osteoblasts, cAMP can be stimulated by PTE.

When cells are isolated enzymatically from the layers of osteoblasts (e.g., of calvaria) and cultured, the cells appear to lose one or more of these characteristics. This makes it necessary to show that the isolated and cultured cells still possess at least potentially all of the properties that define a cell as an osteoblast, if they are to be used to biochemically study the metabolism and hormonal regulation of osteoblasts.

In the present investigation we have succeeded in showing that in OB cell cultures, obtained by isolation of cells from periosteum-free fetal chick calvaria, all osteoblastic properties

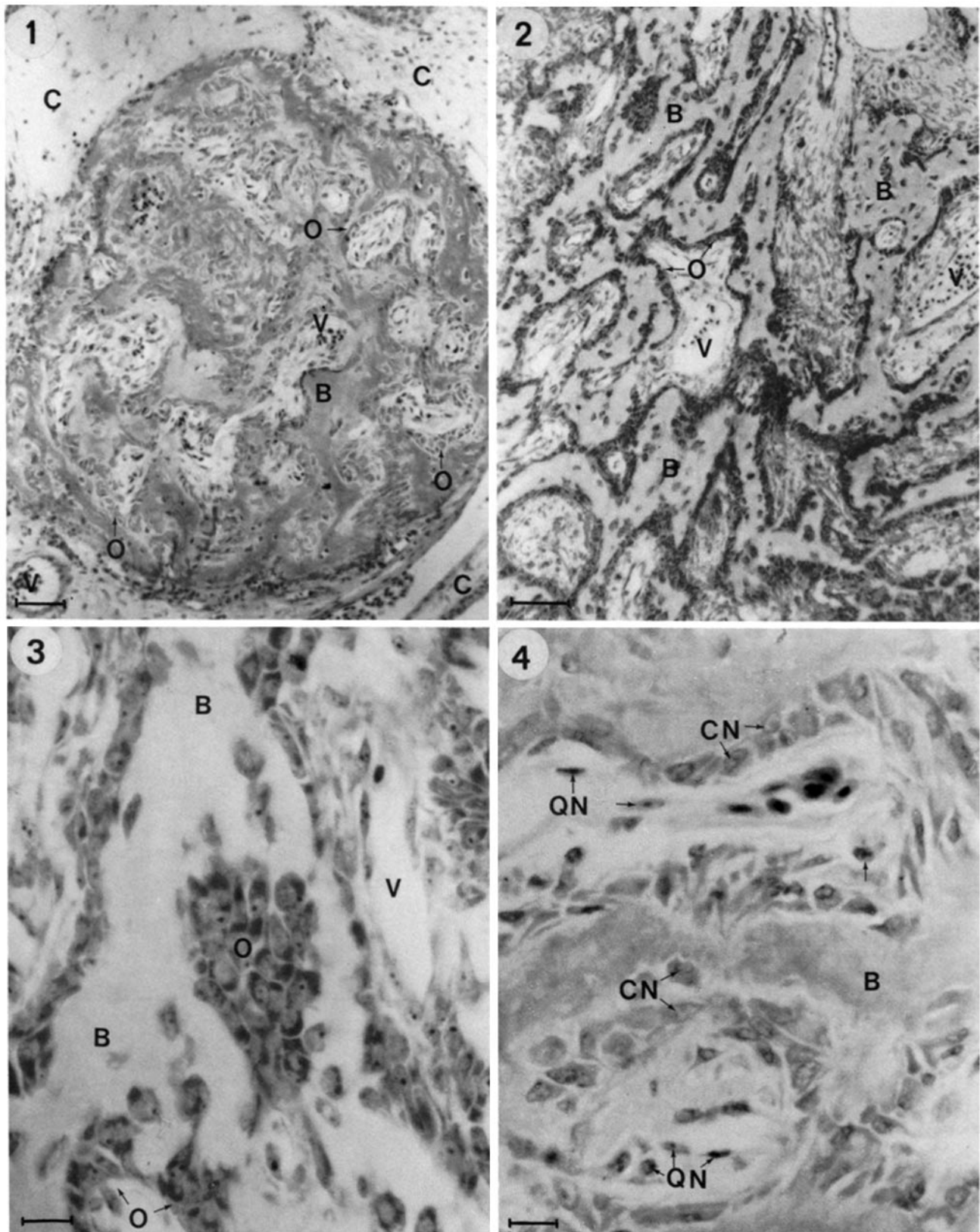


FIGURE 1 OB transplant cultured for 6 d on quail chorio-allantoic membrane. *O*: osteoblast, *B*: bone-like structure, *C*: chorio-allantoic membrane, *V*: blood vessel. Feulgen stain, counter stain: fast green. Bar, 50 μ m. \times 145.

FIGURE 2 OB transplant cultured for 6 d on quail chorio-alloantoic membrane. *O*: osteoblast, *B*: bone-like structure, *V*: blood vessel. Azur-eosin stain. Bar, 50 μ m. \times 200.

FIGURE 3 Detail of Fig. 2. *O*: osteoblast, *B*: bone-like structure, *V*: blood vessel. Azur-eosin stain. Bar, 10 μ m. \times 780.

FIGURE 4 OB transplant cultured for 6 d on quail chorio-allantoic membrane. *B*: bone-like structure, *CN*: chicken nucleus, *QN*: quail nucleus. Feulgen stain, counter stain: fast green. Bar, 10 μ m. \times 780.

are still present after 6 d of culture. This suggests the presence of osteoblasts in the OB cultures although it is not stringently proven that these different properties represent one cell type in the cultures, taking into account that the OB cultures may be heterogeneous. PF cells appear to be fibroblastlike cells lacking these osteoblastic properties.

The biochemical studies reported here give some indication of the purity of the cell cultures, especially of the PF cultures. Since PTH stimulated cAMP production to such high intracellular concentrations in OB cells and had almost no effect in PF cells and since OB cells decarboxylated citrate to such a high degree compared with PF cells, the presence of even a small

percentage of OB cells in the PF preparations would be immediately recognized. The degree of contamination of the OB cell cultures with fibroblastlike cells is more difficult to assess. We are not aware of specific fibroblastlike properties that are

TABLE I
Transplantation Results

		Number of successful transplants			
		present	absent	total	%
OB transplants					
—bonelike structures (azan, azur-eosin, Feulgen)		18	5	23	78
—calcification (von Kossa)		6	4	10	60
PF transplants					
—bonelike structures (azan, azur-eosin, Feulgen)		0	19	19	0
—calcification (von Kossa)		0	12	12	0

Monolayer cultures of OB and PF cells were transplanted on quail chorio-allantoic membrane and cultured for 6 d.

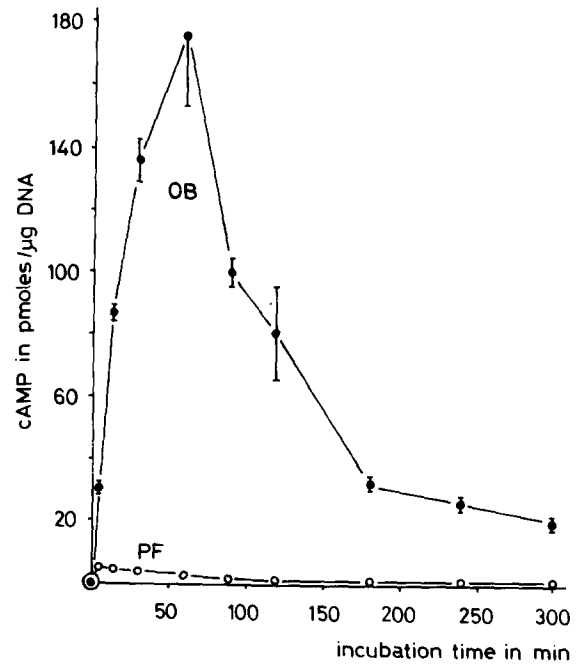


FIGURE 7 Production of cAMP in OB and PF cell cultures in the presence of 1 IU/ml PTH and 1 mM Mix. Values are given as means \pm SE of 6 determinations for OB cultures and of 12 determinations for PF cultures (SE values omitted).

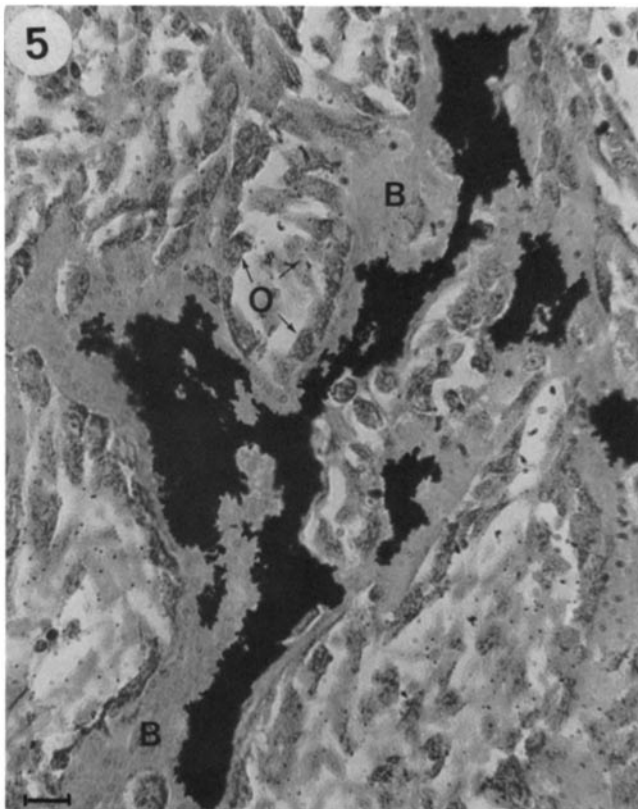


FIGURE 5 OB transplant cultured for 6 d on quail chorio-allantoic membrane. O: osteoblast, B: bone-like structure, black area: mineral. von Kossa stain, counter stain: safranin O. Bar, 10 μ m. \times 500.

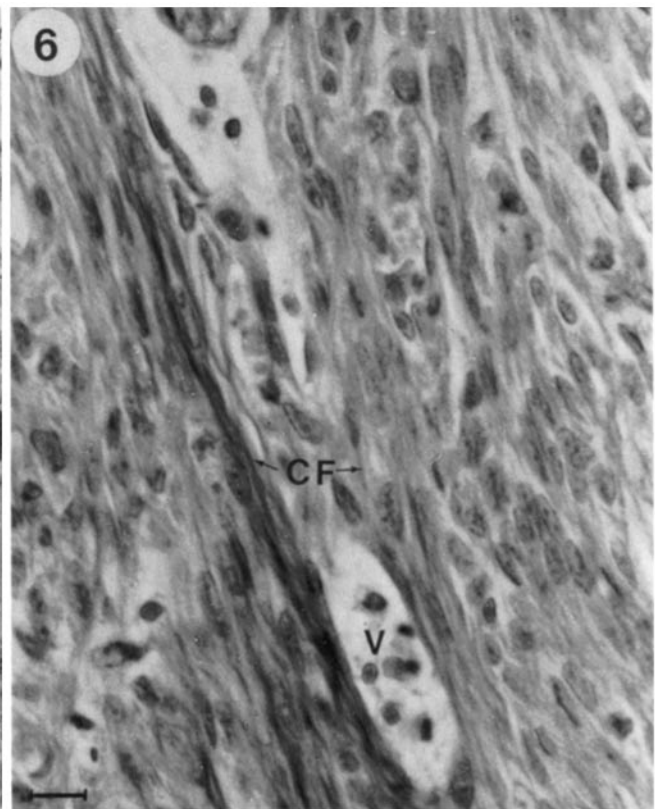


FIGURE 6 PF transplant cultured for 6 d on quail chorio-alloantoic membrane. V: blood vessel, CF: collagen fibrils. Azan stain. Bar, 10 μ m. \times 780.

not present in osteoblasts and could therefore be used to estimate the contamination. Both types of cell cultures may of course be contaminated by other cell types present in calvaria of fetal chicks.

Two other points require discussion. In the first place: in earlier studies (15) we showed that when periosteal membranes are removed from fetal chick calvaria, folded double over the side originally facing the bone matrix, and cultured for a few days, a new generation of osteoblasts was formed. This means that, although osteoblasts do not appear to come along when the periosteum is stripped of the calvarium, the periosteum must contain preosteoblasts or osteoprogenitor cells that can differentiate into osteoblasts (15). Differentiation apparently does not occur when isolated periosteal cells are cultured as monolayer (this study). Either the cell-culture conditions are not favorable for differentiation into osteoblasts, despite being adequate for what are presumably osteoblasts in OB cultures, or the preosteoblasts are completely overgrown by fibroblast-like cells. When transplanted on a chorio-allantoic membrane, PF cultures induce the formation of fibrous tissue, not bone or bonelike structures. This is also surprising in view of the results of for example Ashton et al. (1) who found formation of bone, cartilage, and fibrous tissue when fibroblastlike cultures obtained by culturing marrow stromal cells for 3 to 4 wk were placed in a diffusion chamber and implanted intraperitoneally in adult rabbits. One would expect the cells of fetal periosteal to have similar osteogenic capacities even if preosteoblasts have disappeared during monolayer preculture. An explanation

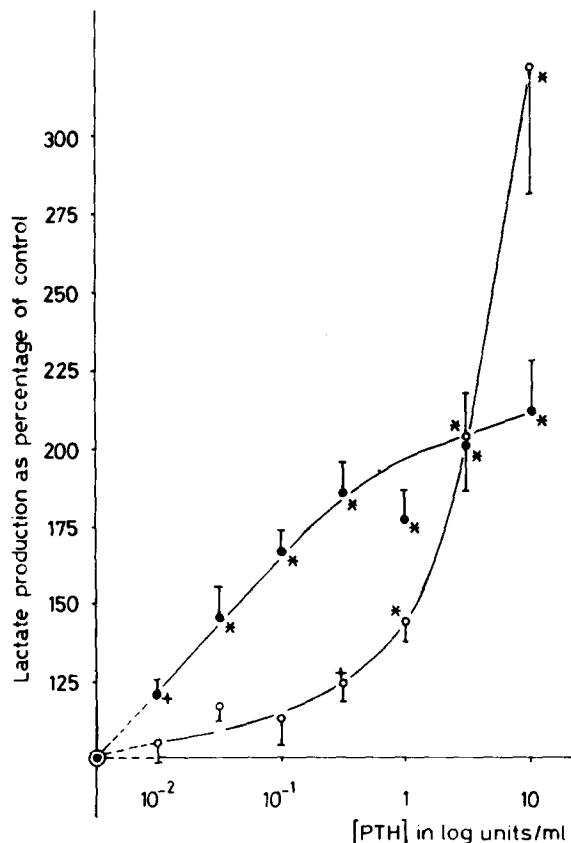


FIGURE 8 Stimulation of lactate production by PTH in OB and PF cell cultures, expressed as percentage of the respective control values. Means \pm SE of 10 (OB) or 8 (PF) determinations. OB control value: $7.91 \pm 0.92 \mu\text{mol/mg DNA}/2 \text{ h}$; PF control value: $9.42 \pm 0.63 \mu\text{mol/mg DNA}/2 \text{ h}$; +: $P < 0.05$, * $P < 0.001$.

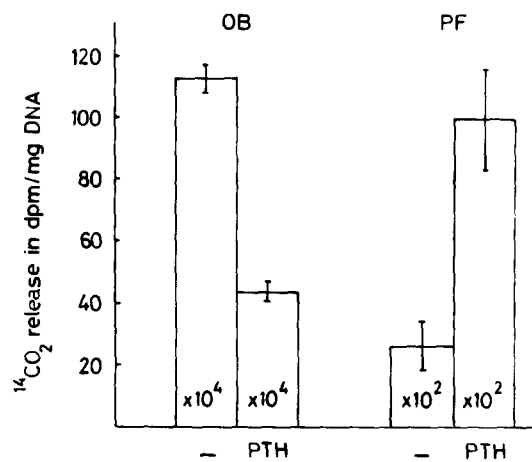


FIGURE 9 Effect of PTH (1 IU/ml) on the decarboxylation of citrate-6-¹⁴C, expressed as amount of ¹⁴CO₂ released in dpm/mg DNA during 2 h of incubation. Means \pm SE of five determinations. OB: $P < 0.001$, PF: $P < 0.01$. Note difference in scale between OB and PF responses.

might be found in the relative short culture time we used for the PF transplants on the quail chorio-alloantoic membrane or in the lack of an inducer needed to stimulate PF cells to differentiate into osteoprogenitor cells and eventually into osteoblasts. If this were the case, OB cells could be considered to be "determined osteogenic cells" (6) in the sense that the cells are in a "competent state of readiness" (11) to form bone if the milieu is favorable. PF cells may be designated as periosteal fibroblastlike cells and possibly inducible osteogenic precursor cells (6).

The second point concerns the conditions under which bone formation and calcification can occur in vitro. In experiments with cultured periosteal membranes (15, 25), bone formation takes place in the center of the culture when the periosteum has been folded double but does not occur on either side of the culture when the periosteum is not folded. Monolayer cultures of osteoblasts in the strict sense of the prefix mono, do not produce bone, but lumps of osteoblasts cultured on CAM appear to do so (this study), although electron microscope corroboration is needed. Even if the mineralization found in "monolayer" bone-cell cultures represents calcified bone formation, this only occurs inside multilayered parts of the cell cultures (19). In other words, bone formation needs a three-dimensional structure. The three-dimensional structure may be essential itself for the stimulation of potential bone-forming cells to produce bone or the three-dimensional structure may be favorable for the creation of a "milieu interieur" from which bone-forming stimulators—perhaps bone morphogenetic proteins (26) secreted by surrounding osteoblasts—cannot easily diffuse away.

This study was supported by the J. A. Cohen Institute for Radiopathology and Radiation Protection, Leiden, The Netherlands.

Received for publication 31 August 1981, and in revised form 30 November 1981.

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