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Differentiation of Osteoblasts and Formation of Mineralized Bone in Vitro

H. C. Tenenbaum and J. N. M. Heersche

Medical Research Council Group in Periodontal Physiology, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada

Summary. Periostea consisting of the osteogenic layer and the fibrous layer of the periosteum were dissected from 17-day-old embryonic chick calvariae, leaving the osteoblasts behind on bone. The dissected periostea were folded with the osteogenic cells in apposition. The explants were cultured on plasma clots for up to 6 days, during which time osteodifferentiation was observed followed by osteoid formation in between the two layers. These cultures consistently mineralized in the presence of 5 or 10 mM β-glycerophosphate. The mineralization and osteoid formation displayed many characteristics identical with those seen in vivo. Specifically, the osteoid formed was birefringent under polarized light, the central zone of osteoid became mineralized within 24 h of formation in vitro, and a clear border between mineralized and nonmineralized osteoid suggestive of a mineralization front was present. The unmineralized osteoid at the periphery was surrounded by osteoblasts. These data suggest that physiologic mineralization of osteoid produced in vitro did occur in this system by the addition of the alkaline phosphatase substrate β-glycerophosphate.

Key words: Mineralization — Osteogenesis — Alkaline phosphatase — In vitro.

To date there have been few studies showing that morphologically distinct osteoid can be formed and mineralized in vitro under cellular control and in a pattern morphologically identical to that seen in vivo. Some investigators have described culture systems in which osteoid formation occurred [1–3], whereas others have developed systems in which both osteoid formation and mineralization could be demonstrated [4–12]. The patterns of mineralization observed, however, were unpredictable and bore little similarity to the pattern of mineralization seen in vivo. Reproducible formation of mineralized bone in vitro was reported by Marvaso and Bernard [13], who have shown that mineralization of osteoid produced by calvarial mesenchyme occurred regularly in a pattern similar to that seen in vivo. This system, however, cannot be manipulated to obtain osteogenesis, osteodifferentiation, or mineralization at will. We describe here a system, adapted from a system first described by Nijweide and Van der Plas [2, 14], that can be used to study in vitro mechanisms which promote and regulate osteodifferentiation and mineralization. Using this system, we have found that osteogenic cells from embryonic periostea differentiate in vitro into osteoblasts and subsequently produce osteoid when cultured folded with the osteogenic layers in apposition. The osteoid formed became mineralized bone when β-glycerophosphate was added to the culture medium. The mineralized bone matrix was surrounded by a clearly distinguishable zone of nonmineralized osteoid.

Materials and Methods

Briefly, periostea consisting of the osteogenic and fibrous layers (Fig. 1A) [30] were dissected from 17-day-old chick embryo calvaria. This procedure effectively removes the fibrous layer and the osteogenic layer of the periosteum while the osteoblasts remain attached to the bone (Fig. 1E). The detached periosteum was then folded double with the osteogenic sides in apposition. The folded periostea were placed on a Millipore filter (AA 0.08 μm) and transferred to a plasma clot consisting of 30% rooster plasma [15], 10% rooster serum, 10% 9-day-old chick embryo extract [7], and 50% Hanks balanced salt solution with or without 5 or 10 mM final concentration of β-glycerophosphate. The clots were prepared and used immediately or were stored at 4°C for a maximum of 24 h. Calculated on the basis of calcium and phosphate values in chicken serum [16] and Hanks balanced salt solution, the calcium concentration in the standard medium is approximately 2.5 mM, the inorganic phosphate concentration approximately 1 mM, and the organic phosphate concentration approximately 4 mM.

Send offprint requests to H. C. Tenenbaum at the above address.

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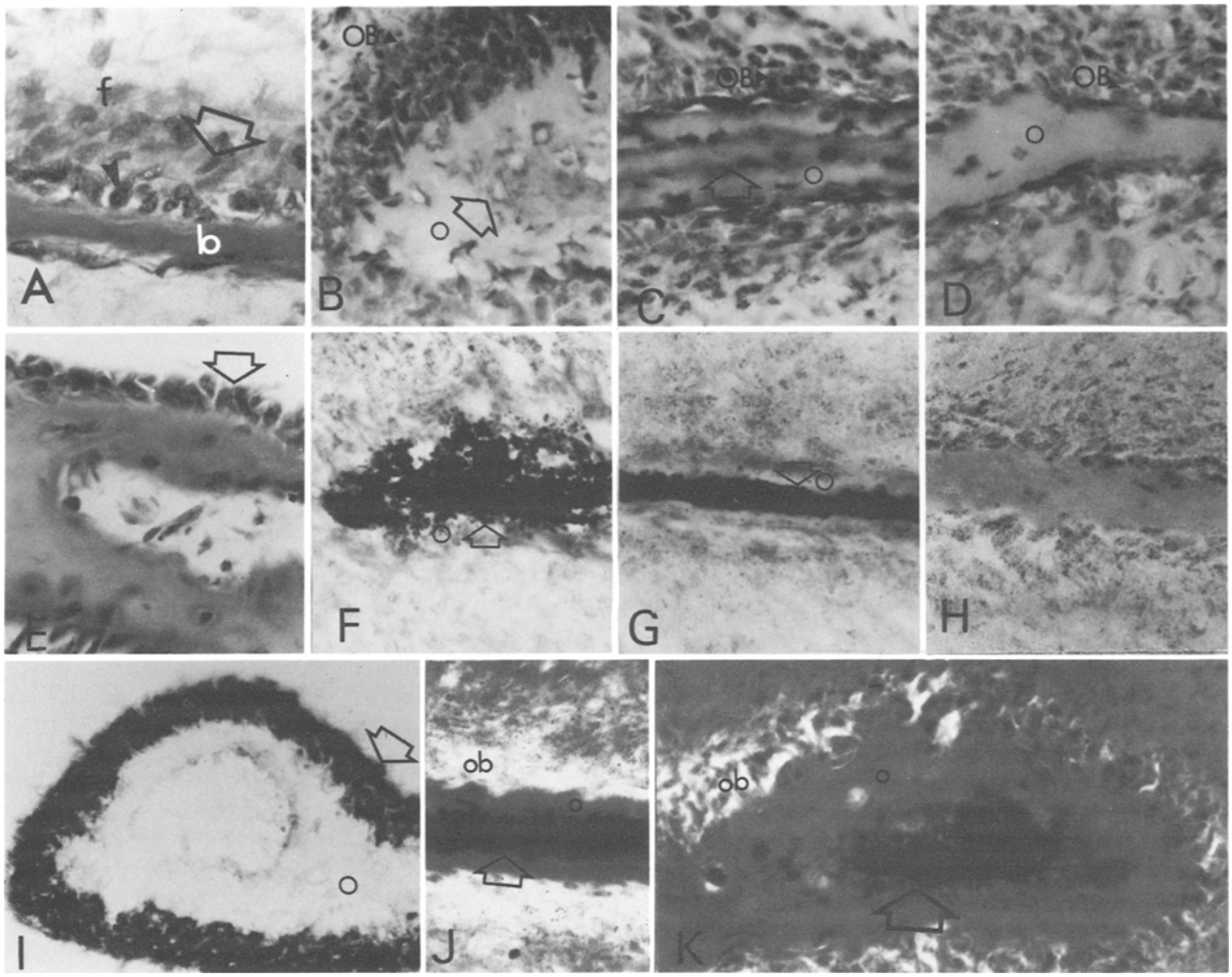

Fig. 1. A Hematoxylin and eosin (H & E) stain of whole 17-day-old chick embryo calvaria. ×398. Note bone (*b*) and the periosteum consisting of osteoblasts (*closed arrow*), osteogenic cells (*open arrow*) and fibrous periosteum (*f*). **B** H & E stain of 6-day culture in presence of 5 mM β-glycerophosphate. ×398. Note border between mineralized and nonmineralized matrix suggestive of a mineralization front (*arrow*), osteoid (*o*), and osteoblasts (*OB*). **C** H & E stain of 6-day culture in presence of 10 mM β-glycerophosphate. ×398. Note clear demarcation of mineralized matrix (*arrow*) and osteoid (*o*) and osteoblasts (*OB*). **D** H & E stain of 6-day culture without added β-glycerophosphate. ×398. Osteoid (*o*), osteoblasts (*OB*). **E** Calvaria of 17-day-old chick embryo after removal of periosteum. ×398. Osteoblasts (*arrow*) left behind. **F** and **G** Von Kossa stain of 6-day culture in presence of 5 mM (**F**) and 10 mM (**G**) β-glycerophosphate. ×398. Dark black stain represents mineralized matrix. Note mineralization border (*arrow*) surrounded by nonmineralized osteoid (*o*). **H** Von Kossa stain of 6-day culture without added β-glycerophosphate. ×398. Note the absence of mineralized matrix. **I** Alkaline phosphatase stain of 6-day culture in presence of 5 mM β-glycerophosphate. ×245. Dark black stain (*arrow*) represents alkaline phosphatase activity in layer of osteoblasts and preosteoblasts (osteogenic cells). Osteoid (o). **J** Von Kossa and van Gieson stain of 6-day culture in presence of 10 mM β-glycerophosphate. ×564. Note mineralization front (*arrow*) surrounded by nonmineralized osteoid (*o*). Clear zone is occupied by osteoblasts (*ob*). **K** Von Kossa, Van Gieson stain and Iron Hematoxylin stain of 6-day culture in presence of 5 mM β-glycerophosphate. ×564. Note mineralization front (*arrow*) surrounded by nonmineralized osteoid (*o*) with osteoblasts (*OB*) on the periphery

Because alkaline phosphatase probably plays a role in mineralization [17–19], β-glycerophosphate was added to the medium as a source of phosphate ions [20] in concentrations of 2.5, 5, and 10 mM. The explants were then cultured for up to 6 days in 5% CO_2 in humidified air at 37°C with changes to new clots every 48 h. Unfixed explants were frozen at various times, sectioned at 5 μm, and stained with hematoxylin and eosin, with the von Kossa stain for calcium salts [21], for alkaline phosphatase activity using AS-MX phosphoric acid as the substrate [22], with the osteoid stain of Ralis and Ralis [23], or with the van Gieson stain [21].

Results

Cultures Without β-Glycerophosphate

On days 2 and 3 of culture, osteoblastic differentiation was observed. By day 4 in culture, osteoid, with osteocytes clearly recognizable, was formed in between the two osteogenic layers of periosteum (Fig. 1D). This osteoid stained positive with the osteoid stain of Ralis and Ralis and negative with the

Table 1. Percentage cultures mineralized in the presence and absence of β-glycerophosphate

β-Glycerophosphate concentration mM	No. of explants	No. of explants with mineralized osteoid	% of Cultures mineralized[a]
0	22	0	0
2.5	4	2	50
5.0	29	29	100
10.0	17	17	100

[a] All cultures, whether mineralized or not, produced osteoid

von Kossa stain for calcified bone matrix (Fig. 1H). Alkaline phosphatase activity was apparent in the osteoprogenitor layer at day 1 and increased continuously up to 6 days in culture (Fig. 1I).

Cultures with β-Glycerophosphate

In the presence of 5 or 10 mM β-glycerophosphate, mineralization of the central zone of osteoid was seen in all cultures (Table 1), while a peripheral zone of osteoid did not mineralize (see Fig. 1B, C, F, G, J, and K). Mineralization with 2.5 mM β-glycerophosphate was unpredictable. If cultures were maintained on β-glycerophosphate-free medium for 4 days and then placed on medium with 5 mM β-glycerophosphate, the pattern of mineralization by day 6 was indistinguishable from that in cultures continuously exposed to 5 mM β-glycerophosphate. Furthermore, sections of mineralized cultures became von Kossa negative after a 10-min incubation in EDTA. Van Gieson staining of sections previously stained by the von Kossa technique demonstrated clear staining of the osteoid surrounding the mineralized osteoid (Figs. 1J, 1K). Finally, the osteoid exhibited birefringency under polarized light.

Discussion

In the present paper, we show that osteoid formed in vitro mineralizes in the presence of an adequate supply of an organic source of phosphate ions. It has been demonstrated before that an appropriate concentration of phosphate ions is required for mineralization in vitro [5]. Furthermore, Raisz and coworkers [24] have shown that as medium phosphate concentrations are increased, osteogenesis is stimulated. In addition, a correlation between blood phosphate levels and osteogenesis in vivo has been established [25]. The rationale for using β-glycerophosphate as an additional source of phosphate ions was that with this reagent, phosphate ions should be made available in places where alkaline phosphatase was present, thereby reducing the possibility of nonspecific mineral precipitation. The localization of the mineral and the presence of a clear border between mineralized osteoid and nonmineralized osteoid strongly suggest that a controlled mineralization process is operating within this system.

A question arises, however, relating to the physiologic basis for raising phosphate concentrations in a medium that has physiologic inorganic phosphate levels to begin with. If in vivo physiologic levels of inorganic phosphate are adequate for the initiation of mineralization, then why are these same inorganic phosphate levels not capable of doing so in vitro? A closer examination of the phosphate concentrations in the culture medium reveals that although inorganic phosphate levels are within the physiologic range (1 mM), the organic phosphate levels (4 mM) are well below physiologic levels (10 mM) [16, 26]. Insofar as the organic phosphates may ultimately be the source of inorganic phosphate through enzymatic hydrolysis by osteoblast phosphatases, a deficiency in their levels might be the limiting factor concerning mineralization. Therefore by raising inorganic phosphate levels above the physiologic range, other investigators [5] may have inadvertently compensated for lower than physiological levels of organic phosphates. Supplementing with an organic phosphate such as β-glycerophosphate might mimic the physiological situation more closely. Although other phosphate sources such as ATP, AMP, ADP, pyrophosphate, and glucose phosphate might be used in the future, evidence obtained in other systems [27] seems to indicate that no demonstrable advantages can be obtained by using these.

In view of the above, it becomes apparent that although inorganic phosphate is required for hydroxyapatite formation, organic phosphates as a source of phosphate ions may be more important than circulating inorganic phosphate in initiation of mineralization. The organic phosphates may thus play an important role in cellular control of mineralization.

An integral part of this hypothesis is the theory put forth by Ramp and Neuman [28] relating to the importance of an intact periosteum as a membrane separating bone and bone fluid (which may contain relatively low levels of organic phosphate) from serum. The periosteal membrane would be able to supply inorganic phosphate for hydroxyapatite formation by enzymatic hydrolysis of serum organic phosphates if and when required. The ambient levels of inorganic phosphates in serum and bone fluid may actually play a relatively small role in the initiation of mineralization.

The osteoid stained positively with the van Gieson stain, which stains collagen fibers deep red

[21]. The birefringency of the osteoid layer further suggests a high degree of fiber orientation, as might be expected for true osteoid. Moreover, when ^{3}H-proline was added to the incubation medium at day 4, autoradiography of sections of 6-day cultures clearly demonstrated proline incorporation in the matrix formed by the osteoblasts (results not shown).

A major stumbling block in the study of bone formation in vitro has been the inability to achieve mineralization of osteoid produced in vitro [29]. The osteoid produced in the present system readily mineralizes upon addition of β-glycerophosphate either from the start of a 6-day culture period or starting at day 4 in culture. The osteoid produced in this plasma-, serum-, and embryonic extract-containing medium therefore seems normal in this respect. It would therefore appear that this culture system can be used to study the mechanisms involved in the control of osteodifferentiation and mineralization in vitro in a way that thus far has not been possible. To be able to more precisely manipulate this system, it would be advantageous to obtain similar results in a completely defined medium. Such experiments are currently in progress [31].

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