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Laboratory Investigations

Differentiation of Osteoid-Producing Cells *In Vitro:* Possible Evidence for the Requirement of a Microenvironment

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

Medical Research Council Group in Periodontal Physiology, Faculty of Dentistry and Mt. Sinai Hospital, Department of Research, University of Toronto, Toronto, Ontario, Canada

Summary: Periostea were dissected from 17-day-old chicken embryo calvariae, placed on millipore filters, and cultured on fluid media containing serum or on serum and plasma containing "plasma clots," in three ways: 1) with the osteogenic layer facing the filter, 2) with the osteogenic layer away from the filter, 3) folded such that the osteogenic layer was in apposition with itself within the fold. The cultures were studied histologically as well as biochemically. Periostea that were cultured folded showed differentiation of osteoblastlike cells after 2 days, and production of osteoid at day 4. Tissues cultured with the osteogenic layer away from the filter demonstrated similar osteoblastic differentiation and osteoid production. Both types of cultures exhibited an increase in histochemically detectable alkaline phosphatase activity over the 4 day culture period that was associated with osteoblasts and the osteogenic area. Periostea cultured with the osteogenic layer facing the filter produced no osteoid. In these cultures, histochemically detectable alkaline phosphatase activity decreased and virtually disappeared over the 4 day culture period. The possibility that the creation of a suitable micro-environment is required for osteodifferentiation in this culture system is discussed.

Key words: Osteogenesis — in Vitro — Microenvironment — Alkaline phosphatase.

Many investigations have been directed to the study of osteogenesis *in vitro*. In particular, studies by Fell [1] and Gaillard [2] illustrated that the periosteum, or cells derived therefrom, could produce osteoid *in vitro*. Later studies by Fitton-Jackson and Smith [3], Goldhaber [4], Binderman et al. [5], and Marvaso and Bernard [6] also demonstrated the *in vitro* skeletogenic potential of periosteal cells.

While these studies demonstrated *in vitro* osteogenesis, the culture conditions and stimuli required for osteodifferentiation and osteogenesis were not analyzed. Others, however, have tried to determine some of the *in vitro* requirements for osteogenesis. Raisz et al. [7] demonstrated that the presence of phosphates enhanced osteogenesis *in vitro*, while Brighton et al. [8] showed that a low oxygen concentration was required in order for osteodifferentiation to take place in cultured periosteum. More recently, Osdoby and Caplan [9] have shown that mesenchymal cells plated at high density became chondrocytic while cells plated at medium density expressed the bone cell phenotype.

In 1975, Nijweide and Van der Plas [10] described a periosteal culture system in which periostea from 18-day-old chick embryonic calvariae formed osteoid when cultured folded with the osteogenic layer of cells on the inside of the fold. When the periostea were cultured unfolded or folded backwards, osteoblasts and osteoid were not observed. This suggested that the folding of the tissue may have provided the conditions required to allow osteogenic cells to differentiate into osteoblasts.

Materials and Methods

Culture System

The explants were prepared from 17-day-old chick embryonic calvariae as previously described [10, 11]. Some periostea were cultured folded with the osteogenic layer of cells on the inside of

Send reprint requests to Dr. H. C. Tenenbaum, Mt. Sinai Hospital, Department of Research, 600 University Avenue, Toronto, Ontario M5G 1X5 Canada

the fold. Others were cultured unfolded, one group with the osteogenic layer away from the medium and another group with the osteogenic layer facing the medium. All explants, maintained at the gas/liquid interface, were cultured at 37°C in 5% CO_2 in humidified air. Medium was changed or explants were transferred to new clots every 48 hours. Samples for biochemistry, light microscopy, and histochemistry were taken at zero, 2, and 4 days. To demonstrate the presence of well mineralized bone, some cultures grown in the presence of B-glycerophosphate and destined for ultrastructural analysis were incubated for 2 more days.

Culture Medium

The solid clot medium consisted of 30% rooster plasma, 10% rooster serum, 10% extract from 9-day-old embryonic chicks, and 50% Hanks balanced salt solution supplemented with $CaCl_2$ to 2.5 mM Ca^{2+} [10]. The blood products and embryonic extract were processed as described by Paul [12]. This medium does not support mineralization and is poorly defined.

In order to use a more defined and reliable medium which was also capable of supporting mineralization, a liquid, serum-supplemented medium was developed. This consisted of BGJ_B medium supplemented with ascorbate 300 μg/ml, 10% fetal calf serum (Gibco), and 10 mM B-glycerophosphate (BGP) (Sigma).

Histology and Histochemistry

The tissues were prepared for hematoxylin and eosin (H&E) staining by fixing in Bouin's fixative and were then embedded in paraffin and cut on a microtome (American Optical) at 5 μm. Specimens destined for alkaline phosphatase enzyme histochemistry were frozen in liquid nitrogen, stored at −20°C, cut on a cryo-microtome (American Optical) at 5 μm, and mounted on glass slides.

Alkaline phosphatase activity was localized using an azo-coupling technique with ASMX phosphoric acid as substrate, as described by Burstone [13]. All stained tissue sections were examined and photographed using a Reichert Univar microscope. Comparisons were made between tissues stained simultaneously under identical conditions. Cultures destined for E.M. were fixed in 2.5% gluteraldehyde, postfixed in osmium tetroxide, and then subjected to routine embedding and sectioning procedures.

Significance of histological results was tested using a Chi square (χ^2) analysis.

Biochemical Measurements

Cultured or fresh periostea were placed in plastic tubes, frozen at −20°C, and kept frozen for up to 1 week prior to assay.

For assay of acid and of alkaline phosphatase activity, the explants were homogenized in 0.5 ml of 3 mM $NaHCO_3$ 150 mM NaCl, pH 7.4 using a polytron homogenizer (Kinematica GMBH, Switzerland). The reaction medium consisted of 60 mM paranitrophenylphosphate in a buffer of 0.37 M 2-amino-2-methyl-1-propanol, pH 10.3. 100 μl of vortexed homogenate was added to 900 μl of the reaction medium. Incubation was carried out at 30°C for 1 hour and was stopped by adding 2 ml of cold 0.25 N NaOH. The amount of liberated paranitrophenol (PNP) was measured as optical density (O.D.) at 410 nm and converted to m moles of PNP. The reaction was linear with time for 3 hours under the incubation conditions used.

Protein content of cultures was determined using the method described by Bradford [14]. Mineralization was quantitated by determining the amount of acid-extractable calcium contained in tissue pellets by atomic absorption (Perkin-Elmer).

Significance of biochemical data was evaluated with the Student's *t* test.

Results

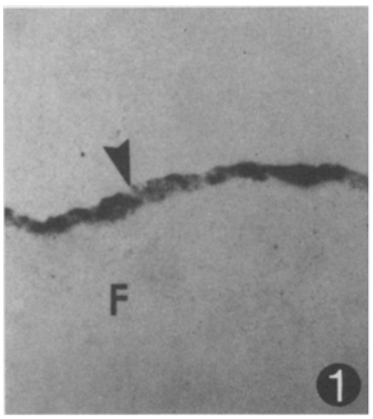
Light Microscopic Histology and Histochemistry

Examination of freshly dissected periosteum failed to reveal the presence of osteoblasts. This tissue consisted primarily of fibroblastlike cells and fibrous connective tissue containing some blood vessels. When this tissue was stained for alkaline phosphatase activity, a small band of positive cells was located in the osteogenic region (Fig. 1). In folded periosteum and in unfolded periosteum cultured with the osteogenic layer away from the medium, a condensation of cells was observed along with an apparent increase in the size of the alkaline phosphatase area, within or near the osteogenic region (Fig. 2). This observation in the osteogenic region was extended in day 4 cultures by the presence of osteoid surrounded by osteoblastlike cells and containing osteocytes (unfolded 9/11 cultures; folded 7/7 cultures) (Figs. 3, 4). The osteoblastlike cells demonstrated an intense alkaline phosphatase stain (Fig. 5).

In contrast, unfolded cultures grown with the osteogenic layer facing the medium did not demonstrate any of the above noted findings (12/12 culture). By day 4 little or no alkaline phosphatase activity could be demonstrated (Fig. 6) and there was a decrease in cellularity coupled with an absence of osteoid (Fig. 7). The difference between unfolded cultures facing the medium and those facing the air phase was tested by χ^2 analysis and found to be significantly different ($P < .01$). In this instance, only osteoid formation was evaluated.

Electron Microscopy

All 6 day cultures destined for electron microscopic evaluation demonstrated bone formation (Fig. 8). The mineralized and nonmineralized matrix surrounds an osteocytelike cell containing a small amount of rough endoplasmic reticulum. The osteoid is bordered by well-polarized osteoblastlike cells which contain extensive rough endoplasmic reticulum.



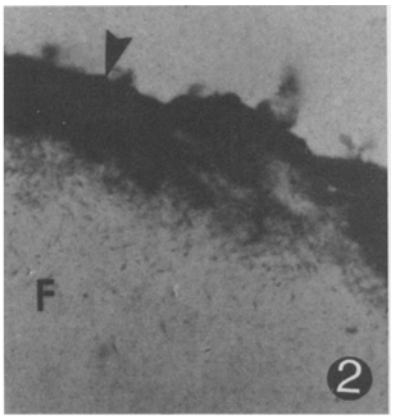
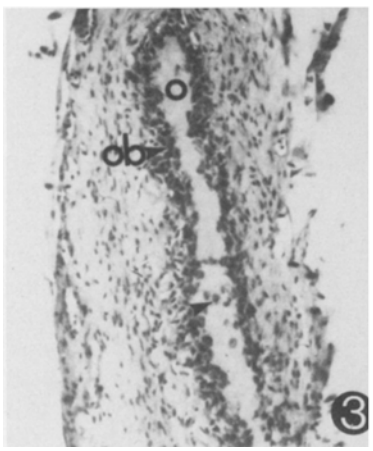


Fig. 1. Alkaline phosphatase stain of 5 μm frozen section of freshly dissected periosteum. Note the presence of staining in the osteogenic layer (arrow) and the complete absence of stain in the fibrous layer (F); ×258.

Fig. 2. Alkaline phosphatase stain of 5 μm frozen section of unfolded periosteum cultured for 2 days with the osteogenic layer of cells facing the air phase. Note the more intense stain within the osteogenic layer (arrow) as compared to fresh (Fig. 1) and absence of stain in the fibrous layer (F); ×414.



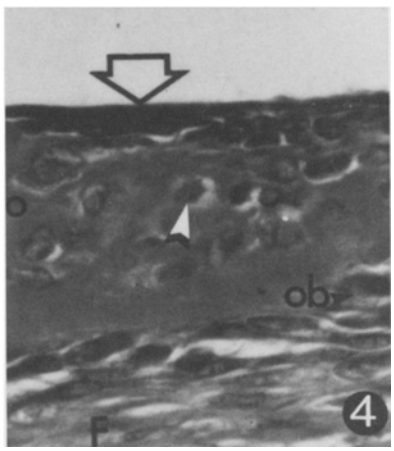


Fig. 3. H & E stain of 5 μm section of folded periosteum cultured for 4 days. Note presence of osteoid (o), containing osteocytes (arrow), surrounded by a multilayer of osteoblasts (ob, arrow); ×258.

Fig. 4. H & E stain of 5 μm section of unfolded periosteum cultured for 4 days with the osteogenic layer facing the air phase. Note the presence of osteoid (o) containing osteocytes (white/black arrow) surrounded by osteoblasts (open arrow).

Biochemistry

Alkaline Phosphatase Activity. Folded cultures and unfolded cultures grown with the osteogenic layer facing the air phase demonstrated initially high alkaline phosphatase activity which subsequently decreased by day 2 to 50% of fresh levels. By day 4 the activity had risen to the levels found in fresh explants (Fig. 9). In contrast, in the cultures grown unfolded and with the osteogenic layer facing the medium, there was a persistent decrease in detectable alkaline phosphatase activity (Fig. 9). This difference was maintained even after normalization of alkaline phosphatase activity against total soluble protein content (Fig. 10a).

Calcium Content

In cultures grown on B-glycerophosphate containing medium it was possible to measure mineral content. This was found to be significantly lower in unfolded cultures grown with the osteogenic layer facing the medium (Fig. 10b).

Discussion

Our results suggest that we can follow the process of osteodifferentiation *in vitro* and that this process appears to be correlated with biochemically or histochemically detectable changes in alkaline phosphatase activity, and calcium accumulation.

Under the proper conditions, cells in the osteogenic layer are capable of differentiating into osteoblasts and producing osteoid which can be subsequently mineralized. Cells were characterized as osteoblasts by their cuboidal morphology, their high AP activity, and their localization adjacent to the osteoid apparently produced by these cells. This argument is supported by electron microscopic evidence confirming the osteoblastic nature of these cells by demonstrating their ability to form a highly structured tissue virtually identical to bone formed in chicken calvariae *in ovo*.

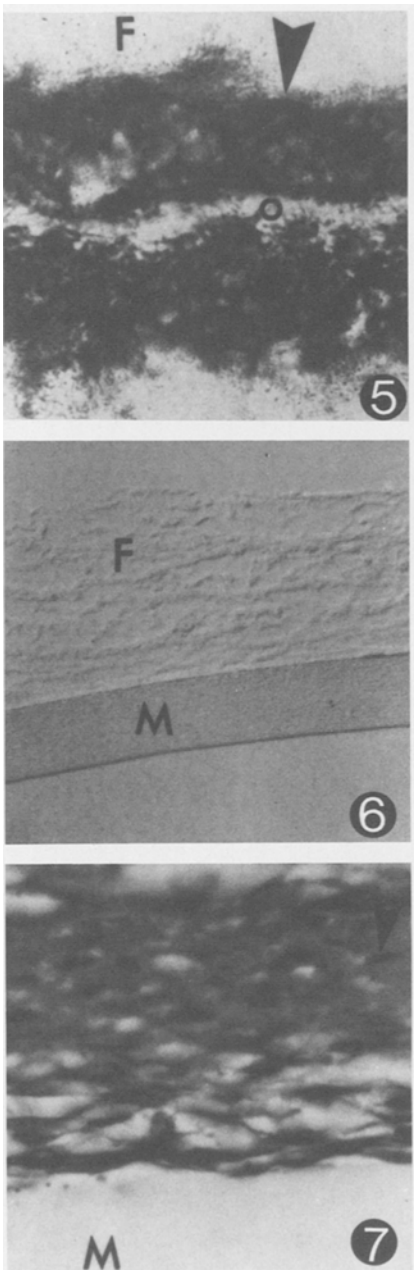


Fig. 5. Alkaline phosphatase stain of 5 μm frozen section of folded periosteum cultured for 4 days. Note intense staining in the osteoblastic layer of cells (arrow) and absence of staining in fibrous layer (F) and in osteoid (o).

Fig. 6. Alkaline phosphatase stain of a 5 μm frozen section of unfolded periosteum cultured for 2 days with the osteogenic layer facing the medium. Millipore Filter (m). Interference contrast optics were used to demonstrate the presence of tissue (fibrous periosteum facing the air phase is indicated with an "F") and to show the complete loss of staining after only 2 days in culture; ×258.

Fig. 7. H & E stain of 5 μm section of unfolded periosteum cultured for 4 days with the osteogenic layer facing the medium phase. Note the absence of osteoid. Few demonstrable cells (arrow). Millipore filter (M).

Fig. 8. Electron micrograph of folded culture grown in B-glycerophosphate-supplemented medium. This picture demonstrates the presence of densely packed collagen cut in cross-section (1) and longitudinally (2). Heavy deposits of mineral (3) can be seen. There is a well-demarcated border between the mineralized and nonmineralized matrix (arrows) which is characteristic of a mineralization front. At the periphery of the unmineralized collagen or osteoid seam are osteoblastlike cells (OB). These contain extensive rough endoplasmic reticulum (4) and are cuboidal. A cell resembling an osteocyte (OC) is surrounded by matrix which is mineralizing. This cell has a less extensive rough endoplasmic reticulum than the active osteoblasts. Nucleus (N), original magnification ×4750.

In our experiments with cultured unfolded periostea, differentiation of osteoblasts was observed when explants were cultured with the osteogenic side facing the air phase while no osteoid developed when similar periostea were cultured with the osteogenic layer facing the culture medium. When tissues are cultured in an organ culture system, a thin film of liquid will form via capillary action over the top of the tissue. It seems likely that, within this film of fluid, even a limited number of cells might condition their surroundings. Therefore, explants cultured with the osteogenic layer of cells facing the air phase might be able to create a microenvironment, whereas, if this layer of cells is

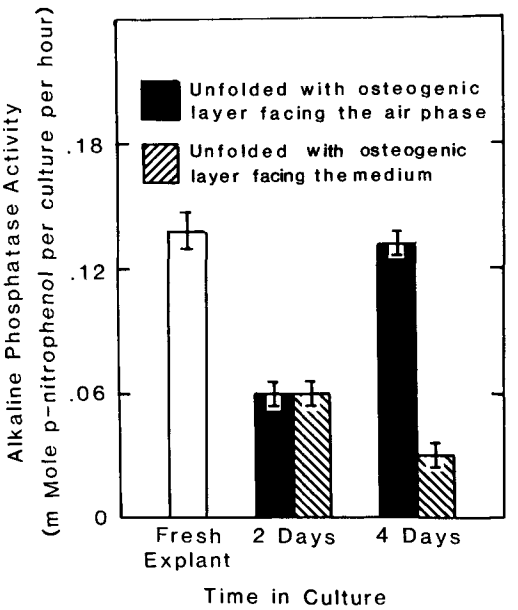


Fig. 9. Alkaline phosphatase activity of cultures after 0, 2, and 4 days of culture on plasma clots. Alkaline phosphatase activity was measured in homogenates of cultures using paranitrophenyl phosphate (PNP) as substrate. Results are expressed in millimoles of PNP degraded per hour per culture. Each bar represents the mean ± SEM of 3 cultures. Analysis was performed using Student's *t* test. At day 4 there was a significant difference between cultures grown with the osteogenic layer facing the air phase and those grown with the osteogenic layer facing the medium ($P < 0.05$). The difference between fresh explants and cultures grown with the osteogenic layer facing the medium is also significant ($P < 0.05$).

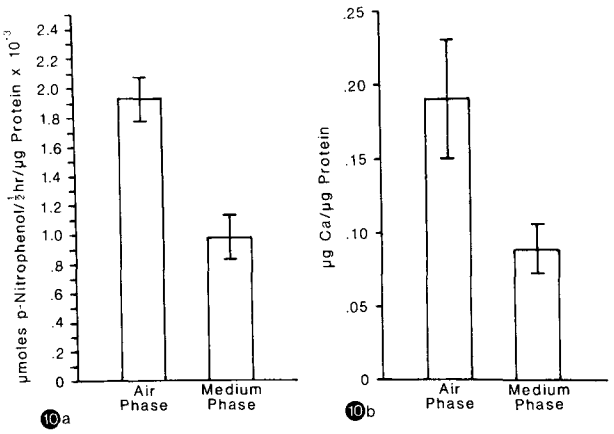


Fig. 10(a). Alkaline phosphatase activity normalized against protein content in cultures grown on BGJ_B medium supplemented with 10% fetal calf serum, 300 μg/ml ascorbate, and 10 mM B-glycerophosphate. There is significantly more alkaline phosphatase activity in unfolded cultures grown with the osteogenic layer facing the air phase ($P < 0.005$). Each bar represents the mean of 8 cultures and the vertical line represents the SEM. **(b)** Calcium content of cultures exposed to B-glycerophosphate (as in Fig. 10a) and grown unfolded with the osteogenic layer facing the medium phase or the air phase. Cultures grown with the osteogenic layer facing the air phase contained significantly more calcium than cultures grown with the osteogenic layer of cells facing the medium phase ($P < .05$). Each bar represents the mean of 8 cultures and the vertical line represents the SEM.

cultured facing the culture medium, they would be under "diffusing" conditions and thus not able to create a microenvironment.

The observation that osteoid develops within the fold of explants cultured with the osteogenic layer on the inside of the fold might similarly be related to the establishment of an environment where factors important for osteodifferentiation are accumulating. A microenvironment, thus, might allow for the concentration of factors produced by cells that could in turn stimulate complete differentiation of those cells. Alternatively, factors accumulating in the microenvironment might inactivate specific differentiation inhibitors present in the serum-, plasma- or embryonic extract-component of the culture medium in analogy with the process taking place in myogenic differentiation *in vitro* [15, 16]. Similar results obtained using serum-free, chemically defined medium (data not shown) suggest that the differentiation patterns are similar to those observed in the serum-, plasma- and embryonic extract-containing medium and therefore this possibility would seem unlikely.

Clearly, the evidence presented here for the role of a microenvironment is circumstantial in nature. Other possible factors related to differentiation would include oxygen tension changes [8, 17] or cell density changes [9] when periosteal tissues are cultured in their various configuration. We have not tested these possibilities.

Recent data obtained using selectively permeable Diaflo membranes instead of millipore filters have supported the microenvironment hypothesis, however. In the presence of the appropriate pore size, even cultures grown with the osteogenic layer facing the medium produced bone [18] and (Tenenbaum HC, Smith I, Heersche JNM, Palangio K, unpublished data). These results would suggest that putative osteoinductive factors are being trapped by the Diaflo membranes just as factors might be trapped in the microenvironment created by folding or culturing unfolded with the osteogenic layer facing the air phase.

In summary, our results are compatible with the view that the osteogenic cells of the periosteum are capable of differentiating within their immediate environment when the periosteum is cultured folded or with the osteogenic layer facing upward. It may be possible that a microenvironment might promote osteodifferentiation because of the sequestration of

osteoinductive factors. Similar phenomena could play an important role in the stimulation of differentiation of osteoblasts *in vivo*.

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