MINERALIZATION OF GROUND SUBSTANCE OF NEW BONE TISSUE IN MOUSE MARROW ORGAN CULTURES

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Fragments of bone marrow form bone tissue when transplanted in vivo [4, 5]. In vitro, however, osteogenic differentiation of marrow tissue has not yet been successfully obtained. A cultural system was developed recently in which fragments of adult mouse bone marrow form loosely woven bone tissue [2], very similar in its morphological, histochemical, and ultrastructural characteristics to native bone tissue as found in the body [3].

The final stage of osteogenic differentiation in this system, namely mineralization of the ground substance of the newly formed bone, takes place only if the culture medium contains sodium glycerophosphate which, under the influence of the enzyme alkaline phosphatase, releases inorganic phosphate, which then binds with calcium ions to form insoluble hydroxyapatite. By varying the times of addition and removal of glycerophosphate, it is thus possible to study the temporal parameters of deposition and mineralization of the ground substance of bone tissue, which can be done only with difficulty by studying osteogenesis in vivo.

EXPERIMENTAL METHOD

Bone marrow was flushed out of the femora of (CBA × C57BL)F_1 mice weighing 18-22 g. The marrow was cultured on the boundary between two phases by the multiple organ culture method [1]. Bone marrow from femur, in the form of the whole fragment, was placed on an HAWP Millipore filter (pore diameter 0.45 μ, thickness 150 μ), shaped like a square with sides 8 mm long. The culture medium consisted of 80% of MEM medium in the α-modification, 20% embryonic calf serum, and the following additives: vitamin C (0.15 mg/ml), glucose (4 mg/ml), L-glutamine (0.5 mg/ml), and penicillin and streptomycin (60 U/ml of each). The material was cultured at 37°C in an atmosphere consisting of a mixture of air with 5-7% CO_2, and saturated with water vapor. The culture medium was changed twice a week. After 10 days or later sodium β-glycerophosphate was added to some of the cultures in a final concentration of 10 mM. At subsequent changes of medium, depending on the scheme of the experiment, the same concentration of glycerophosphate was either added or not added. The cultures were fixed with 96% ethanol. Some cultures were embedded in paraffin wax, and serial sections cut from them; others were used to obtain total preparations. The material was stained with alum hematoxylin, for alkaline phosphatase by Gomori's method, and for insoluble calcium salts by Von Cossa's method.

EXPERIMENTAL RESULTS

In the first week of culture hematopoietic cells and macrophages migrated from the explant on to the filter, and this was followed by development of a fibroblastic zone of growth, which gradually widened and thickened [2].

By the end of the second week of culture foci of compaction had formed in the fibroblastic layer, and the cells in them became round or polygonal in shape and began to lay down ground substance. As they joined together these foci of deposition of ground substance assumed the shape of bone trabeculae. Both cells and ground substance of these trabeculae showed high alkaline phosphatase activity by Gomori's method.

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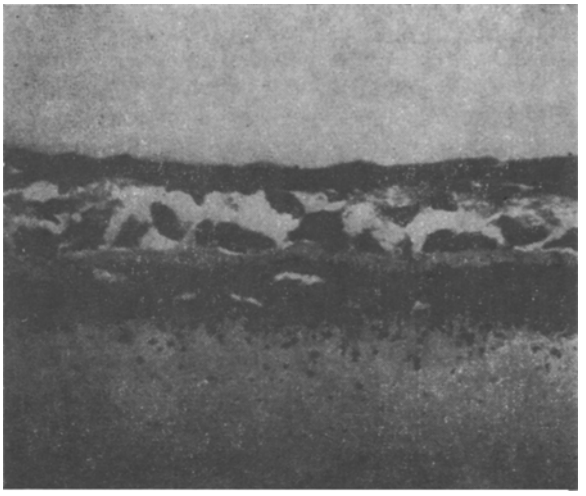

Fig. 1. Mouse bone marrow culture, section, 19th day of culture, sodium glycerophosphate present from 16th to 19th days. Here and in Figs. 2 and 3: Von Cossa's reaction, hematoxylin, 400 ×.

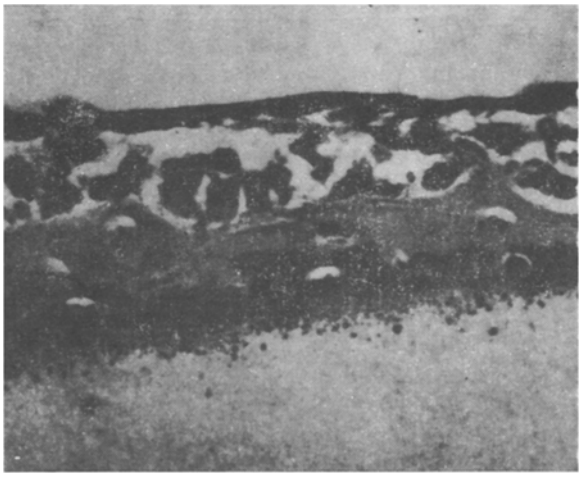

Fig. 2. Mouse bone marrow culture, section, 22nd day, sodium glycerophosphate from 16th to 19th days.

Toward the 20th day of culture much of the filter was covered by a stratified cellular structure. In transverse section these cultures had a characteristic structure. Immediately next to the filter as a rule there was a layer of osteoblasts, whose processes penetrated deeply into the pores of the filter. Above them lay a thick layer of ground substance, several tens of microns thick, containing cavities with osteocytes immured in them. Higher still were one or more layers of polygonal osteoblasts, covered above by layers of spindle-shaped fibroblasts.

If sodium glycerophosphate was present in the culture medium after the 10th day of culture the whole thickness of the ground substance of the newly formed bone tissue, with the exception of a narrow border of ground substance in direct contact with the upper layer of osteoblasts, was found to be impregnated with insoluble calcium salts; after Von Cossa's reaction this appeared in the form of a dense black deposit of silver grains. Calcium salts also seeped into the Millipore filter to a depth comparable with the depth of penetration of the cell processes (several tens of microns). If sodium glycerophosphate was not present in the medium the cultures developed on the whole similarly, but no mineralization of the ground substance was observed.

Intensive deposition of ground substance of new bone usually began on the 16th day of culture. In the cultures of group 1 sodium glycerophosphate was added on the 16th day, and

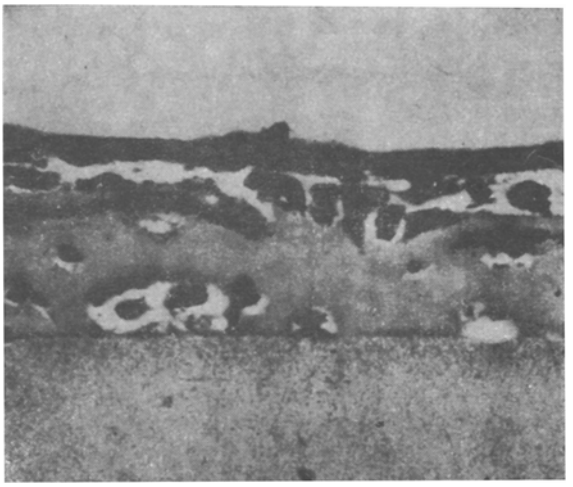

Fig. 3. Mouse bone marrow culture, section, 23rd day, sodium glycerophosphate present from 19th to 23rd days.

after the 19th day they were replaced in medium without glycerophosphate. In these cultures, fixed on the 19th day, insoluble calcium salts penetrated throughout the thickness of the ground substance except in a narrow zone in immediate contact with the upper layer of osteoblasts (osteoid). The upper part of the Millipore filter also appeared to be mineralized (Fig. 1).

When these cultures were fixed on the 22nd-23rd day of culture, not the whole layer of ground substance appeared to be impregnated with insoluble calcium salts, but only its lower (basal) part, accounting in different preparations for between 1/3 and 3/4 of the whole thickness of the ground substance. The upper layers of ground substance remained unmineralized (Fig. 2).

In 26-day cultures an even smaller part of the ground substance was mineralized, namely that adjacent to the filter — from 1/3 to 1/5 of its thickness or less. In this case the intensity of mineralization was reduced compared with cultures fixed on the 19th day: calcium remained most abundant in the most basal regions of the ground substance. The zone of the filter adjacent to the ground substance, as before, contained calcium salts.

Cultures of the second group were kept on medium with sodium glycerophosphate starting from the 19th day of culture. When fixed on the 21st day most of the ground substance remained unmineralized. Only in the upper, apical regions of the ground substance was a narrow zone of calcification present, resembling a black strip, only very slightly thicker than the unmineralized osteoid lying immediately above it.

When the material was fixed on the 22nd-23rd day the zone of calcification had spread to more extensive regions and was a little thicker, but as before it occupied only a very small part of the ground substance as regards both its length and its thickness (Fig. 3). At later times of fixation (26-30 days), despite the presence of sodium glycerophosphate in the medium, the regions of calcification had not widened, and the intensity of mineralization could even have fallen. By these times of culture the deposition of new ground substance was evidently coming to an end already, for calcium salts incorporated previously continued to be excreted. Deposition of insoluble calcium salts into the filter was not observed in the cultures of group 2.

Thus if sodium glycerophosphate was present in the culture medium, the ground substance of the new bone tissue underwent mineralization. However, not all the ground substance had this capability. Evidently only freshly synthesized ground substance of bone tissue can incorporate calcium salts. Meanwhile the youngest ground substance (osteoid) is not mineralized.

The ground substance in the cultural system described above was deposited in an upward, apical direction relative to the filter and, consequently, the upper layers of osteoblasts, lying above the ground substance, took part in its formation. Osteoblasts of the lower layer, whose processes penetrated into the pores of the filter, were evidently responsible for mineralization of the filter itself, but only in the early stages (not after the 19th day) of culture.

Mineralization of the ground substance in the cultures examined was limited in time (it began on the 16th day and was completed on the 23rd-26th days after explantation); in different experiments this period could be shifted a little one way or the other. Cessation of deposition of the ground substance and of its mineralization in the later stages of culture was probably connected with worsening of the nutrition of the bone cells as the culture grew thicker.

The impression was obtained that when sodium glycerophosphate was absent from the culture medium (and even when it was present in 26- to 30-day cultures, insoluble calcium salts deposited previously in the ground substance could be excreted from it, and the later the ground substance was formed, the more easily this took place. Whether osteoblasts were necessary for this process, for without them remodeling of bone tissue in vivo has not been described, remains unclear. In some cultures the zone of growth contained giant multinuclear cells, but when glycerophosphate was excluded, bursts of hematopoiesis were observed on the surface of the upper fibroblastic layer. However, the nature and functions of these multinuclear cells have not yet been explained.

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