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THE DEVELOPMENTAL FEATURES OF MARROW STROMA IN ECTOPIC BONE MARROW IMPLANTS

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Abstract

Implantation of bits of marrow in ectopic sites is followed by reorganization of tissue and the formation of a hemopoietic nodule surrounded by a shell of bone. This regenerative process is reminiscent of marrow ontogeny and the model can serve to study marrow ontogeny in a relatively short period of time. Early events during this regeneration were studied by scanning (SEM) and transmission electron microscopy (TEM). Within 24 hours the implant elicited an angiogenic reaction and new vessels penetrated the implant. Intense circulation, thus established, divested the implant from hemopoietic cells, leaving the stroma behind. Stromal cells proliferated and the impetus for this proliferation appeared to result from an impulse caused by the presence of bony fragments outside and within the stromal cells. Previous studies of this model have not appreciated the presence of non-viable bone in the implant, although the fact that non-viable bone can trigger osteogenesis and new bone marrow formation is well-known. This experimental model lends itself to the study of the interrelationship of hemopoietic cells and their supporting stroma as well as the interrelationship of bone and hemopoiesis.

Key words: Hemopoiesis, bone marrow, marrow ontogeny, marrow stroma, marrow development, marrow growth, marrow implant, extramedullary hemopoiesis, mesenchymal tissue, bone formation, bone resorption.

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Introduction

When bits of marrow tissue are removed from the marrow cavity and implanted in ectopic sites, a sequence of events is followed leading to the repair of tissue and establishment of a new functioning hemopoietic tissue surrounded by a shell of bone (6). The sequence of events associated with this repair process, which recapitulates marrow ontogeny, (10,11) has been studied by light and electron (7,10,16) microscopy. This process is similar to the regenerative process after ablative curretage of marrow cavity (3). The repair process originates from stromal cells of marrow which dedifferentiate into primitive mesenchyme. Part of this mesenchyme then redifferentiates into osteoblasts and begins to lay down osteoid tissue which then calcifies and forms osteoid bone. Within the interstices of this osteoid tissue, a primordial marrow cavity is formed and the stromal structure of marrow is reorganized. The primordial marrow is then seeded by hemopoietic progenitor cells. Hemopoietic proliferation follows and leads to the resorption of bone, excepting a peripheral shell. The result of this process is an ectopic hemopoietic nodule surrounded by a shell of bone.

In the present study, using scanning (SEM) and transmission (TEM) electron microscopy, we have studied early events during this process to learn how this repair process is initiated.

Materials and Methods

Male Sprague-Dawley rats (200-250 g) were used in all experiments. The technique for subcutaneous implantation has been described in detail previously (4). Briefly, under intraperitoneal anesthesia using sodium pentobarbital (1 mg/kg BW) an incision was made at the knee. A hole was then drilled in the articular surface of the femur using a low speed dental drill. A polyethylene tube (gauge 16) was inserted into the entire length of the marrow cavity. The free end was clamped with a hemostat and the tube, now containing marrow tissue, was removed. Through a small incision in the skin of the abdomen a pocket was made in the subcutaneous tissue. One end of the polyethylene tube was then fitted to a needle attached to an empty syringe and the other end was placed in the subcutaneous pocket. The marrow was slowly deposited in the pocket and the

incision was closed.

After 1,3,5 and 7 days, the incision was opened and the implants were removed and placed into either 1% glutaraldehyde buffered with sodium cacodylate (pH 7.2) or modified Karnovsky's solution similarly buffered. Fixation was done overnight at 4°C. The implants were then cut into small blocks of about 1-2 mm and post-fixed in 1% similarly buffered OsO_4 for 45 minutes. The blocks were then dehydrated in graded ethanol. For SEM, the tissue was critical point dried in liquid CO_2 and sputter-coated with gold-palladium and studied in JEOL 100 CX TEM Scan. For TEM, the dehydrated tissue was embedded in Epon 812, and thick sections were obtained with glass knives, stained with paragon multiple stain and screened with light microscope. Desirable sections were then trimmed and thin-sectioned with diamond knives, stained with uranyl acetate and lead citrate and studied in a JEOL 100 CX TEM Scan.

Results

At 24 h, the marrow tissue was divested of most hemopoietic cells so that the stromal network, usually difficult to observe in fully functioning marrow, was clearly visible (Fig. 1). Few hemopoietic cells were left; most free cells within the tissue consisted of red cells. The stromal network appeared in SEM as a spongelike structure displaying many interconnecting chambers from which hemopoietic cells had been depleted (Figs. 1 and 2). The stromal meshwork itself consisted of cellular and acellular elements. Acellular elements were comprised of fibrillar structures and bony fragments that were generated during the drilling of the femoral epiphysis. The cellular component of stroma (Fig. 3) consisted of cells with indistinct borders and nuclei containing large amounts of euchromatin with slight peripheral condensation and generally a large nucleolus. Some of these cells were elongated while others were rounded. The latter could have originated from the former through a modulation process or, alternatively, they could have been a different cell type altogether. The common denominator of all stromal cells was the presence of numerous profiles of rough endoplasmic reticulum (RER) displaying distended cisternae (Fig. 3). In addition numerous dark, small round mitochondria were observed. Many stromal cells appeared to contain bony fragments within vacuoles in their cytoplasm (Fig. 3). However, the presence of lysosomal structures was not a common finding in these cells and the cells did not appear overtly phagocytic. Numerous bone fragments were also seen in the extracellular space. The tissue as a whole contained numerous small blood vessels.

By day 3, the tissue was now more cellular and denser. Newly formed vessels were prominent, having developed an adventitial layer, and very few free red cells were seen within the tissue (Fig. 4). Proliferating stromal cells gave a monotonous feature to the tissue. Large fragments of apparently non-viable bony tissue were still present in the implant. They were in association with what might be osteoclasts (Fig. 5). Proliferating stromal cells contained numerous profiles of RER, and many free ribosomes, resembling osteoblasts and suggesting differentiation into bone forming tissue. In fact, the lay down of osteoid tissue was evident in certain areas (Fig. 6). The most striking finding, however, was the presence of numerous interdigitating surface microvilli in stromal cells. These microvilli occasionally reached 1-2 micrometers in length. In addition, other inflammatory cells such as mast cells and eosinophils, normally seen in granulation tissue, were also observed.

By day 5, the stromal cells had retained their monotonous features, but they were now more elongated and some contained fewer microvilli (Fig. 7). They still contained bony fragments that were now in the process of disintegration.

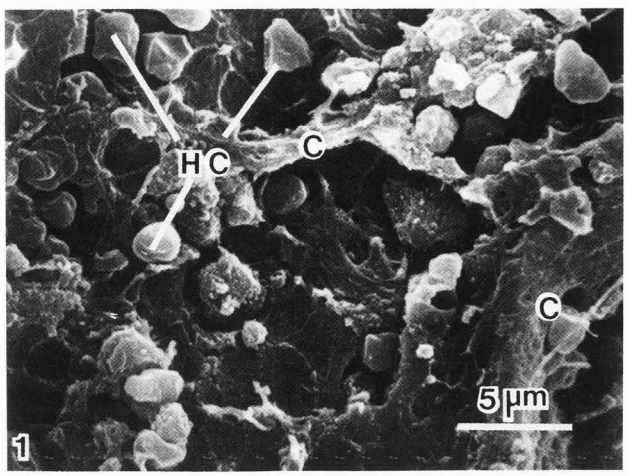
Osteoblastic transformation was even more evident on day 7 when numerous morphologically typical osteoblasts were seen forming a dense tissue (Fig. 6) interspersed with loose areas rich in small blood vessels and stromal cells that were elongated or stellate-shaped and contained far less RER as compared to osteoblasts. Collagen deposition was prominent in the areas containing osteoblasts (Fig. 6).

Discussion

Growth and regeneration of ectopic marrow implants, in their essential features, is highly reminiscent of marrow ontogeny and it has been emphasized that this model recapitulate marrow's ontogeny in a relatively short period of time (10,11). Since the study of marrow ontogeny is rather difficult in the fetus, the ectopic marrow implants may serve as a convenient experimental model to study the ontogeny of marrow.

Various aspects of regeneration of marrow in light microscopy as well as SEM and TEM has been described previously (6,7,10,11,16) and the sequence of events described here is consistent with previous findings. In all these studies, however, the implant has been considered to contain only marrow tissue without bone. The impetus to the regenerative sequence is hence considered unknown. The present study documents the presence of bony fragments in the marrow implant. These bony fragments are generally non-viable tissue produced during the drilling of bone. They are subsequently seen in association with marrow stromal cells, either intracellular or extracellular (Figs. 3 and 5). We propose that the non-viable bony fragments might provide the necessary impetus for the regeneration process, and proliferation of stromal cells and their subsequent differentiation into osteoblasts. Consistent with this interpretation are several works indicating that non-viable, decalcified bone and tooth matrix are able to induce osteogenesis in the recipient tissue (1,2,5). However, in our experimental model, the regeneration process appears to have its origin in the donor tissue (4,13).

In addition, the present work indicates that implantation of marrow bits is associated with rapid angiogenesis which revascularizes the implant. This is due to the fact that marrow has a high potential for production of angiogenic factors (7). In this regard, marrow is in contrast to such other tissues as kidney (12). and liver (14) that do not exhibit this potential. Ectopic implants of liver and kidney, therefore, undergo necrosis of "coagulation type:



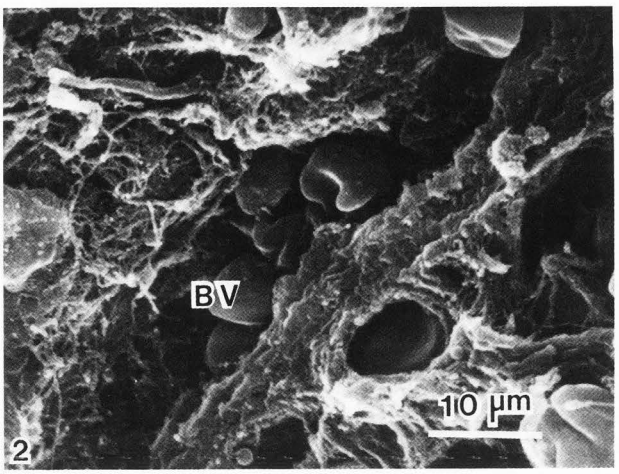
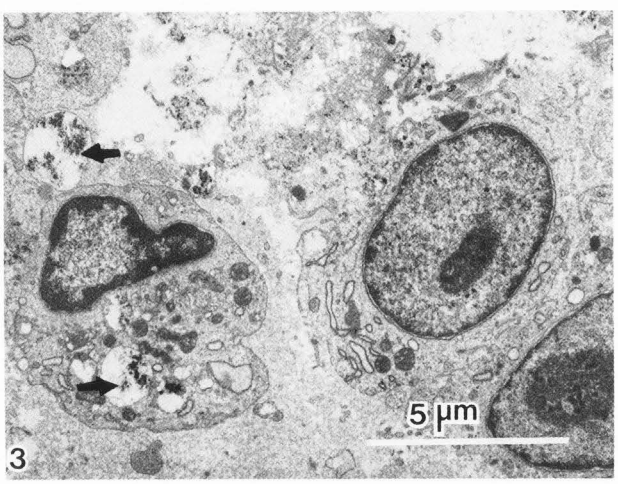


Fig. 1. SEM of marrow implant after 24 h. Most hemopoietic cells have left the implant leaving the stromal network clearly visible, although still a few hemopoietic cells are seen (HC). This network consists of interconnecting cords (C), sponge-like in appearance, interspersed with few red cells and few remaining hemopoietic cells.

Fig. 2. SEM of marrow implant on day 3. An invading blood vessel (BV) runs diagonally and contains numerous red cells. The stroma consists of a fibrocellular meshwork, divested of hemopoietic cells.

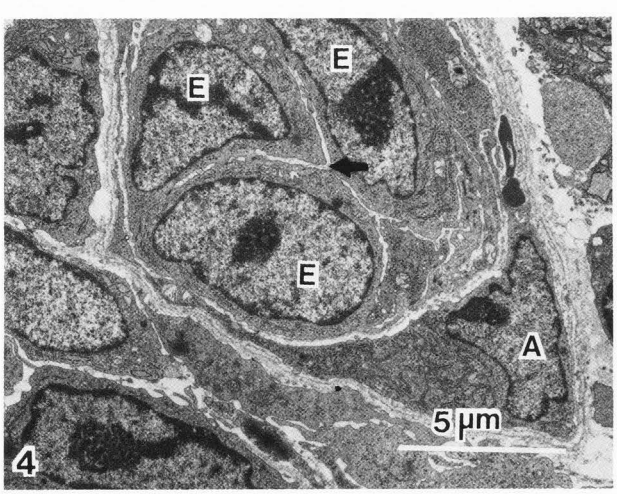
Fig. 3. TEM of marrow implant after 24 h. Two stromal cells are seen. One of them contains bony fragments in the intracellular vacuoles (Arrow). These fragments may provide impetus for the subsequent dedifferentiation of cells into primitive mesenchyme.

Fig. 4. TEM of implant on day 3. An invading blood vessel dominates the figure. Its high endothelial cells (E) have left the lumen (arrowhead) almost obliterated. An adventitial cell (A) covers the endothelial layer.



indicating lack of vascularization (12,14). Even spleen, in this regard, is far inferior to the marrow because it elicits angiogenesis only after 3 days (15,8,9).

The rapid angiogenesis that the marrow tissue exhibits within a few hours, insures the survival of tissue in the ectopic site. Invasion of implants by newly-formed blood vessels and the establishment of an intense circulation within the tissue during the first 24 h leads to the divestiture of most hemopoietic cells, leaving the stromal cells interspersed with these newly invading small blood vessels. Because these small blood vessels lack the wall structure of established capillaries, some bleeding in the tissue is inevitable and this explains our observation on the presence of free red cells within the stromal tissue. In general. this phase of regeneration resembles the formation of granulation tissue after injury.



Further study of this model may elucidate the relationship between the hemopoietic tissue and its supporting stroma as well as the interrelationship of bone and marrow.

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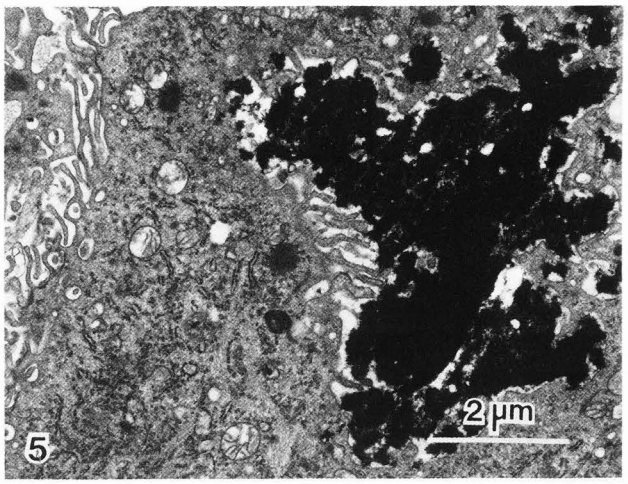


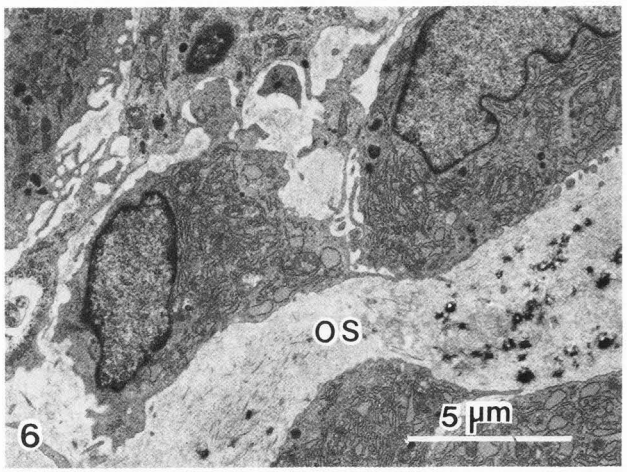
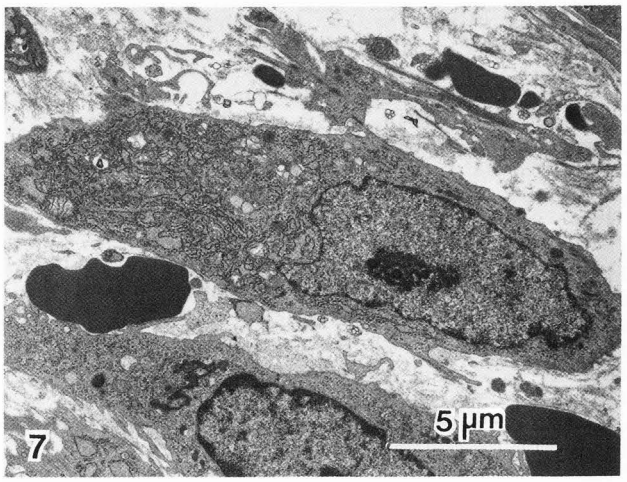


Fig. 5. TEM of implant on day 3. Part of a cell is seen of which the salient feature is the presence of numerous long interdigitating microvilli. The cell is in association with a large bony fragment.

Fig. 6. Implant on day 7. Osteoid tissue (OS) is recognized by the presence of several osteoblasts displaying prominent features of protein synthesis. Extracellular osteoid fibers run diagonally in the low part of the figure.

Fig. 7. TEM of implant on day 5. The primordial marrow cavity is seen as a loose connective tissue with 2 elongated stromal cells that contain abundant profiles of RER, nuclei containing mostly euchromatin and a single nucleolus. In the intercellular space fibrous structures are seen interspersed with cytoplasmic cellular processes and few red cells free in the tissue.



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Discussion with Reviewers

W.H. Knospe: Have you considered whether lysis of bone during histogenesis may induce a mesenchymal cell to develop into a stromal cell capable of supporting hemopoiesis? Does bone contain a factor capable of inducing such a stromal cell? The studies of Reddi et al. (Proc. Natl. Acad. Sci. USA 72: 2212, 1975) and Knospe et al. (Int. J. Cell Cloning 3: 320, 1985) suggest that it does.
Authors: We agree. The relation of bone to hemopoiesis is actually a relatively unexplored area. But there is ample evidence that resorption of bone may stimulate hemopoiesis probably by providing stromal microenvironment supportive of hemopoiesis. For instance Van Dyke (Clin. Orthop. 52: 37, 1967) has found a remarkable similarity between the distribution of blood flow in the bone and the distribution of erythropoietic marrow in the skeleton. Little (Gerontologia 15: 155, 1969) has also found that active hemopoiesis is seen in those bones where resorption is active. But the exact mechanism whereby this relation comes about deserves further studies. The model we have described here may actually serve to elucidate their relationship.

T.M. Seed: The dominant "stromal" cell-type (shown in Fig. 5) has a number of features strikingly similar to the osteoclast-like cells shown at later times of implant. Would the authors like to comment on the possibility that the dominant day-3 stromal cells, with pronounced interdigitating microvilli, are osteolytic monocytic cell types, directly related to the multinucleated osteoclasts?
Authors: This is indeed possible; although the cell shown in this figure, and commonly seen on day 3, lacks numerous lysosomes the presence of which is a characteristic feature of osteolytic monocytic cells. It is, however, possible that we may be dealing with a cell type in different functional states.

F. Campbell: Since many macrophages must have been present in the implanted marrow, and since one might expect macrophages or monocytes to migrate into this tissue, it surprises me that no macrophages were identified in these implants. In this same vein, are macrophages present when this tissue resumes hematopoiesis? Also, the cell on the left in figure 3 seems to me to have many of the features of a macrophage.
Authors: Macrophages are indeed common in these implants, particularly in the primordial marrow cavity that develops one week after implantation. Whether these macrophages develop from mesenchymal cells or they migrate into the implant is not known. The cell in fig. 3 may resemble a macrophage because it contains some bone, but it lacks lysosomes that are the hallmark of macrophage cell system. Therefore the cell cannot be identified as a macrophage.