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The cartilaginous fracture callus in rats

Anders Henricson
Anders Hulth
Olof Johnell

Lund University Department of Orthopedics at Malmö General Hospital, S-214 01 Malmö, Sweden

The right tibia was broken manually in 56 rats weighing 100 g; the fracture was stabilized with an intramedullary steel wire. Groups of rats were killed after 3-30 days. The fracture with its surrounding musculature was dissected out and immediately frozen to −70° C. Cryostat sections of the fracture region were stained with hematoxylin/eosin, toluidine blue, and immunoenzymatically with collagen II antibodies. Another series of 30 fractured rats were killed after 1-15 days, and the fractures were examined histologically after decalcification with EDTA.

Two types of callus were observed. The periosteal-endosteal callus started as proliferation of preosteoblasts without inflammatory cells on Day 1 and developed bone trabeculae from Day 3 until Days 8-10, but not thereafter. The cartilaginous callus was formed by condensation of fibroblastlike mesenchymal cells mixed with inflammatory cells outside the periosteal callus and started on Day 5 at the fracture fragments denuded of periosteum. The cells differentiating to cartilage seemed to migrate from the surrounding musculature and its newly formed vessels. The enchondral bone formation started close to the periosteal callus from which vessels were piercing into the then hypertrophic and mineralized chondrocytes on Day 11.

We conclude that the bridging callus is formed by fundaments of periosteal callus derived from predetermined cells and the bridge of enchondrally formed bone trabeculae by cells migrating from outside.

Introduction

The formation of cartilaginous callus in the repair of displaced experimental fractures is an almost regular feature. However, cartilage appears only in the external callus and not in the calluses that are formed by osteoprogenitor cells. The origin of external callus is not completely understood (McKibbin 1978), and there are two main theories.

The dominating theory suggests that the osteoprogenitor cells in the periosteum, together with stem cells in the bone marrow, also form external callus (Ham & Harris 1972, Kernek & Wray 1973, Owen 1980). Formation of cartilage is believed to depend on an insufficient vascular supply and low oxygen content in the rapidly growing callus tissue (Ham & Harris 1972). The motion of the fragments is also said to promote the formation of cartilage instead of bone (Pritchard 1964).

On the other hand, many authors have proposed that the cells forming the external callus derive from the surrounding musculature or from newly formed sprouting vessels (Trueta 1963, Pritchard 1964, McLean & Urist 1968); it is not clear whether the cells come from the muscles, vascular endothelium, or from cells in the blood stream. Brighton (1984) suggested that the mesenchymal cells from the outside become induced and differentiated in the fracture area into chondroblasts that later undergo enchondral bone transformation.

We describe the extension and topography of cartilaginous callus and its resorption and replacement by bone tissue.

Materials and methods

Fifty-six Sprague-Dawley rats of both sexes, weighing 100 g at the beginning of the experiment, were used. Under mebumal anesthesia, a thin flexible steel wire (0.4 mm in diameter) was inserted into the bone marrow of the right tibia from a small hole at the tuberosity. The tibia was then manually broken. Groups of 4 to 10 rats were killed after 3, 5, 8, 11, 15, and 30 days. The fracture

region, with surrounding musculature, was dissected out, immediately frozen in isopenthane, and stored at −70° C. 8 μm cryostate sections were made and stained immunoenzymatically with hematoxylin as a background. Antibodies against Ia-antigen and different subsets of T lymphocytes were used (Hulth et al. 1985, Hulth et al. unpublished data). Three different antibodies reactive with three different epitopes on the human collagen Type II were tested. The antibodies were generated in DB A/1 mice after immunization with native chicken collagen. Only one of the antibodies, the C II–II antibody, reacted with the cartilage in the fractures; a detailed characterization of the antibodies and the assay system has been presented elsewhere (Klareskog et al. 1986)

Another series of 30 rats were fractured in the same way and two rats were killed daily. The specimens in this series were fixed in neutral formalin, demineralized in EDTA, embedded in paraffin, sectioned to a thickness of 8 μm and stained with hematoxylin and eosin. These sections served as a reference when studying the frozen sections, which were not always maintained undamaged due to difficulties in cutting sections containing both hard and soft tissues.

Staining for alkaline phosphatases was done on the undecalcified frozen sections after fixation in formalin for 10 min, incubation in naphtol-AS-Tr, and staining with red violet diazo salt. Other sections were stained with toluidine blue at neutral pH.

Results

The formation of periosteal callus was seen on Day 2 and developed for 8–10 days, but not thereafter. From Day 2–3, the proliferating osteoblasts in the periosteum formed bone trabeculae without passing an intermediate stage of cartilage. The same was true of bone formed in the orifice of the medullary canal (sealing callus). External callus developed mainly outside periosteal callus, starting on the side of the tibia that abutted the musculature (Figure 1).

From Day 2, it was possible to see scattered mesenchymal cells in all the free spaces, mainly between the muscle fibers that were separated from each other. On Day 3, crowding of cells, such as macrophages, mesenchymal cells, and lymphocytes, was visible outside the periosteal callus (Figure 2) and most of all close to the fracture ends that were denuded of periosteum and the rest of the hematoma. This area of crowded irregular cells, i.e., *the area of pluripotent cells*, widened backwards from the fracture ends, forwards to bridge the fracture, and outwards. Chondroblasts apparently differentiated from the pluripotent cells, and this differentiation started on Days 4–5 at the denuded fracture ends, first on the muscle side and then on the skin side of the fracture (Figure 1).

The area of the cartilage widened very rapidly, and on Day 8, most of the free space on the muscle side of the fracture and even parts of the musculature were occupied by cartilage. Smaller amounts of cartilage also developed in the fracture gap. The marrow canal was sealed by osseous trabeculae.

Remnants of the pluripotent cells could be observed outside the cartilage even after 30 days. During the first week, a distinct boundary be-

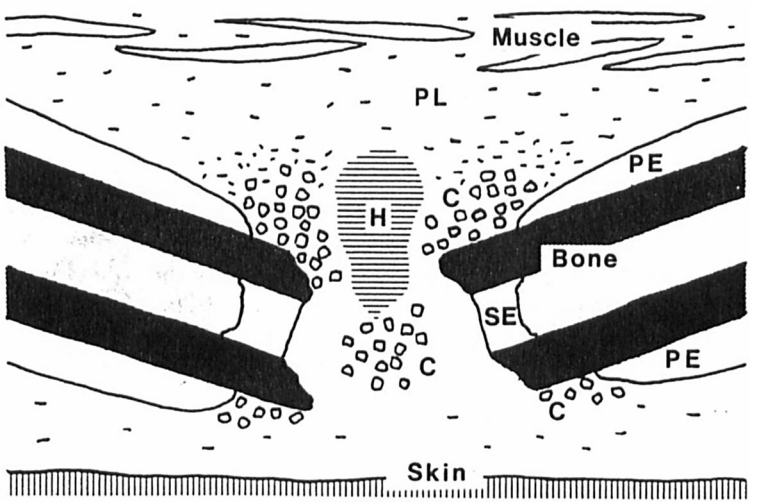

Figure 1. A drawing of the fracture healing on Day 5. Most of the pluripotent cells (PL) develop on the muscle side of the fracture. Direct bone formation occurs from the periosteum (PE) and also in the orifices of the bone marrow canals as sealing callus (SE). Cartilage formation (C) starts at the denuded fracture ends and between the fragments. The hematoma is marked H.

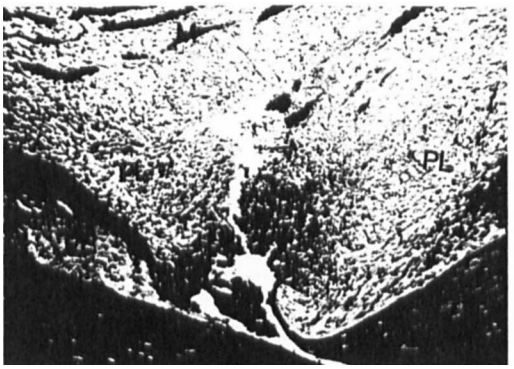

Figure 2. Fracture healing on Day 3. The two fragments (B) are visible. In the middle, the rest of the hematoma (H). Periosteal callus (PE) has developed on both sides, with bone formation to the left. The area from the periosteal callus towards the muscle fibers (M) is filled with cells that become crowded near the periosteal callus. This mass of cells (PL) contains numerous capillaries, HE, ×63.

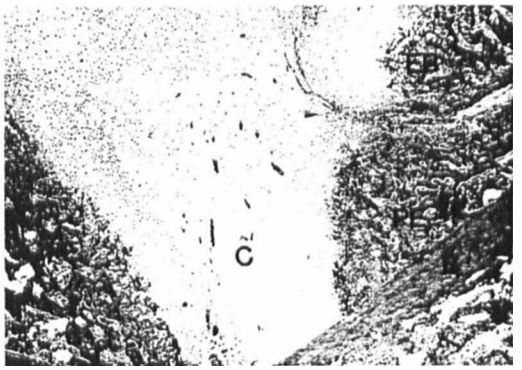

Figure 3. Fracture healing on Day 12. Cartilaginous callus (C) in the form of a V. The fractured bone (B), periosteal callus (PE) with buds of capillaries on the surface, enchondral bone formation (EB). HE, ×25.

tween the periosteal callus and the cartilaginous external callus was observed.

On Days 8–9, the chondrocytes facing the periosteal callus became hypertrophic and obtained a mineralized matrix. One or 2 days later, buds of capillaries extended from the periosteal callus towards the hypertrophic chondrocytes (Figure 3). The vascularity of the spaces between the periosteal bone trabeculae provided with active osteoblasts increased. Vessel canals piercing the cortical bone from the bone marrow towards the new ossification front increased in number and width. Resorption of hypertrophic chondrocytes occurred and the subsequent osteoblasts formed bone on the cores of the cartilaginous matrix.

The enchondral bone formation occurred on both sides of the fracture and between the fragments as well; the entire process seemed to depend mainly on vessels originating from the marrow canal, the periosteal callus, and from the enchondral bone as it was formed. The vascularization of the interfragmentary enchondral bone formation originated also from the bone marrow and from the sealing callus. The front of enchondral bone formation proceeded faster at some distance from the fracture than close to it, resulting in the typical V-form (Figure 3) of the remaining cartilage with the apex of the V pointing towards the fracture line on about Days 12–15. A typical feature was also extended straight vessels from the interfragmentary area into the center of the cartilage, initiating bone formation there after resorption of the chondroblasts.

Both the nuclei and the cytoplasm of the osteoblasts of the periosteal callus stained for *alkaline phosphatase*. The chondrocyte nuclei were also positive. Cells in the area of pluripotent cells were also stained, particularly in the zone adjacent to the cartilage, where even the cytoplasm of the pluripotent cells was stained. A part of the intense staining in this area was certainly due to the occurrence of vascular endothelium in newly formed vessels.

Toluidine blue stained the cytoplasm and the matrix of the cartilage metachromatically. The same was true for a majority of the pluripotent cells, in particular those close to the cartilage. Some of the mononuclear cells between the muscle fibers were also stained metachromatically. *Mast cells* existed outside the fracture area in the musculature, subcutaneously, and scantily in the area of pluripotent cells, but never in the cartilage. The mast cells were numerous around small vessels and capillaries (Figure 4).

Labelling with anticollagen II antibodies occurred only in the cartilaginous callus from Day 5. From the beginning, only limited areas of the cartilage became stained to a brownish color, mostly in the periphery of the cartilage. On Day 11, most of the new cartilage was stained (Figure 5). It was not a uniform staining, but areas with intensive staining were mixed with those with weaker staining.

Figure 4. Fracture healing on Day 15. Mast cells close to capillaries outside the area of the pluripotent cells (PL) staining metachromatically. Toluidine blue, ×25.

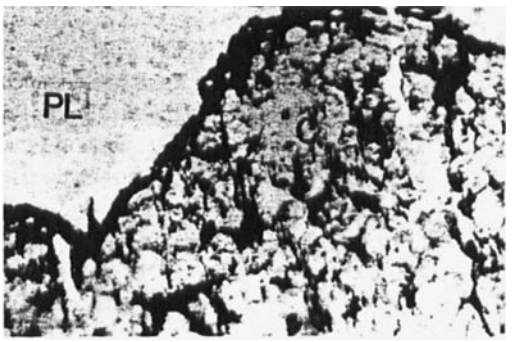

Figure 5. Fracture healing on Day 11. Cartilage (C) is stained with antibodies against collagen II, but not the area of the pluripotent cells (PL). ×160.

Discussion

In an earlier paper (Hulth et al. 1985), we described the development of the pluripotent cells consisting of fibroblastlike mesenchymal cells and inflammatory cells, such as Ia-presenting cells and different subtypes of T lymphocytes. The area of pluripotent cells is exuberant on the muscle side of the fracture and less on the skin side. Apparently, the cartilage of the callus differentiates from these cells. The direct bone formation in the periosteal callus and in the marrow originates from a more homogeneous cell mass, the preosteoblasts, which does not contain inflammatory cells. The function of the inflammatory cells in the differentiation process, if any, is not clear. The presence of these cells at the formation of cartilage, however, discriminates the enchondral pathway from the direct bone formation. Therefore, it seems likely that cartilage does not develop as an alternative to direct bone formation in fracture repair, but that each tissue has its own territory and origin. The maximal development of the cartilaginous callus on the muscle side of the fracture makes it tempting to suppose that the origin of the mesenchymal cells is to be found in the musculature or in the new vessels formed in it. There is a connection between bone induction and muscle, as demonstrated by Buring & Urist (1967): demineralized matrix develops into cartilage and bone when it is put together with a piece of muscle in a millipore chamber. Nathanson (1985) has recently emphasized the close connection between skeletal muscle and chondrogenesis in the presence of bone morphogenetic protein (BMP). Reddi & Huggins (1972) demonstrated that powder of acid-soluble matrix, implanted subcutaneously in rats, transforms into cartilage on the 5th day and in bone on the 11th day, and that this transformation occurs from the muscle side of the implanted material. It should be observed that in our experiments the timetable for the development of cartilage and bone, respectively, was exactly the same as in the experiments of Reddi & Huggins.

The formation of cartilage from the pluripotent cells is not necessarily due to an insufficient vascular supply and a low oxygen pressure in the area of these cells. It is known from other types of cartilage that this tissue, in order to keep its integrity, is itself able to regulate its optimal oxygen milieu by producing a vessel-repelling substance (Sorgente & Dorey 1980). During fetal and postfetal life, most of the skeleton is developed through enchondral bone formation, and external inductive callus is truly a repetition of this course (Reddi & Anderson 1976).

As the cartilage formation develops from the 5th day and onwards, the immunoenzymatic reaction indicating the production of collagen II becomes more and more intensive. This collagen does not, to any high degree, become masked by proteoglycan, as occurs in intact joint cartilage (Klareskog et al. 1986).

Metachromatic staining with toluidine blue was

apparent not only in the cartilage, but even in the area of the pluripotent cells regularly adjacent to the cartilage and also in certain fields at a distance from the cartilage. Cells around muscle fibers also produce a metachromatic substance, possibly hyaluronic acid (Maurer & Hudack 1952), but during the further differentiation into chondrocytes, the pluripotent cells most likely produce other types of glycosaminoglycans. Mast cells were present mostly adjacent to small vessels outside the area of fracture repair, and also in sparse groups among the pluripotent cells, but never in the cartilage. The occurrence of the mast cells in callus formation is described by Lindholm et al. (1969). Their relationship to small vessels is apparent, suggesting a role in angiogenesis.

The visible callus in clinical experience and in animal experiments (Mølster et al. 1982) is larger in nonoperated fractures than in exactly reduced and rigidly fixed fractures, indicating the importance of mechanical factors stimulating external callus. Through supply of cartilaginous callus from the outside, nature has solved the problem of bone repair in unstable fractures. In rigidly fixed fractures, bone repair is brought about by direct membranous bone formation through osteoprogenitor cells, giving a sparse external callus. A sufficient supply of external cartilaginous callus in unstable fractures is probably dependent on relatively intact surrounding soft tissues (Hulth 1980, Whiteside & Lasker 1978). This also accords with the clinical experience that fractures caused by direct high-energy trauma with damage to muscles, as a group, heal more slowly than fractures caused by indirect trauma (Edwards 1964).

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