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Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone

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SUMMARY

We examined immunohistochemically the fracture repair process in rat tibial bone using antibodies to PCNA, BMP2, TGF-β 1,-2,-3, TGF-β R1,-R2, bFGF, bFGFR, PDGF, VEGF, and S-100. The peak level of cell proliferation as revealed by PCNA labelling appeared first in primitive mesenchymal cells and inflammatory cells at the fracture edges and neighboring periosteum at 2-days after fracture, followed by the peaks of periosteal primitive fibroblasts and chondroblasts, which appeared at fracture edges at 3- and 4-days after fracture, respectively. BMP2 was weakly positive in primitive mesenchymal cells, osteoblasts and chondroblasts. At 3-days post-fracture, periosteal osteoblasts produced osteoid tissue and callus with marrow spaces lined by osteoblasts and osteoclasts, and all primitive mesenchymal cells and osteoblasts were positive for TGF-β 1,-2,-3, and TGF-β R1,-R2. They were also positive for vascular growth factors bFGF, FGFR and PDGF, but negative for VEGF, and the peak of PCNA labelling of vascular endothelial cells in the marrow space was delayed to 4-days after fracture. Chondroblasts at fracture edges produced hypertrophic chondrocytes at 5-days after fracture and they were positive for TGF-β 1,-2,-3, and TGF-β R1,-R2. Primitive chondroblasts were positive for vascular growth factors VEGF as well as bFGF, FGFR, and the peak of PCNA labelling of vascular endothelial cells in the cartilage was at 5-days after fracture. Hypertrophic chondrocytes were also positive for these growth factors but negative for bFGF and bFGFR. S-100 protein-induced calcification was only positive on chondroblasts and hypertrophic chondrocytes. At 7-days after fracture, bone began to be formed from the cartilage at fracture edges, by a process similar to bone formation in the growth plate. Enchondral ossification established a bridge between both fracture edges and periosteal membranous ossification encompassed the fracture site like a sheath at 14-day after fracture. Our study of fracture repair of bone indicates that this process is complex and occurs through various steps involving various growth factors.

INTRODUCTION

Many growth factors are considered as critical regulators of fracture repair of bone (Joyce *et al.*, 1990, Jingushi *et al.*, 1991, Thorp *et al.*, 1992, Andrew *et al.*, 1995, Horner *et al.*, 1996, Iwaki *et al.*, 1997, Opperman *et al.*, 1997, Horner *et al.*, 1998).

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In the initial stages of bone fracture repair, mobilized macrophages produce various growth factors, including fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factor (TGF)-β (Baird *et al.*, 1985, Shimokado *et al.*, 1985, Assoian *et al.*, 1987), which stimulate the proliferation of fibroblast-like primitive mesenchymal cells (Ross and Benditt, 1961) and their subsequent transformation and differentiation to chondroblasts (Bourque *et al.*, 1993).

Basic FGF (bFGF) has been identified in primitive chondrocytes, chondroblasts and osteoblasts as a stimulator of their proliferation and bone formation by membranous and cartilaginous ossification, respectively (Bolander, 1992). bFGF is also known to stimulate endothelial cell replication and neovascularization (Gimenex-Gallego *et al.*, 1986, Montesano *et al.*, 1986, Canalis *et al.*). PDGF liberated also from the platelet granules (Shimokado *et al.*, 1985), and damaged vascular endothelial cells (Majesky *et al.*, 1990), is a potent stimulator of proliferation and differentiation of chondroblasts, osteoblasts and their precursors (Graves *et al.*, 1989, Pfeilshifer, 1990, Skoog *et al.*, 1990, Joyce *et al.*, 1991, Tsukamoto *et al.*, 1991, Zhang *et al.*, 1991, Bourque *et al.*, 1993, Andrew *et al.*, 1995, Horner *et al.*, 1996). TGF-β 1,2,3 is also secreted from platelet granules (Assoian *et al.*, 1989), and fracture-exposed bone matrix (Hauschka *et al.*, 1986), and stimulates bone formation by regulating the formation and function of chondrocytes, osteoblasts and osteoclasts (Robey *et al.*, 1987, O'Keefe *et al.*, 1988, Hock *et al.*, 1990, Horner *et al.*, 1998). Bone morphogenetic protein (BMP) was initially discovered as a factor that induced ectopic bone formation (Urist *et al.*, 1952, Urist, 1965, Urist *et al.*, 1971) and later demonstrated to induce the proliferation and differentiation of primitive mesenchymal cells to chondroblasts and osteoblasts(Yamaguchi *et al.*, 1991, Katagiri *et al.*, 1995). BMP is also known to initiate the cascade production of other growth factors during osteogenesis (Reddi, 1995). These growth factors are also produced by osteoblasts and chondrocytes and are highly concentrated in skeletal tissues (Hauschka *et al.*, 1986, Kim *et al.*, 1996).

Gerber *et al.* (1999) recently demonstrated that the vascular endothelial growth factor (VEGF) is expressed in hypertrophic chondrocytes but not in resting or proliferating ones. VEGF is a key regulator of angiogenesis necessary for enchondral ossification. Several investigators have found that S-100 protein contains binding sites specific for Ca^{2+} (Manabe *et al.*, 1981, Takahashi *et al.*, 1984). Weiss and Dorfman (1986) indicated that the calcium binding properties of S-100 protein, which is present in chondrocytes, are related to the mechanisms involved in matrix calcification. This relationship stems from the localization of intensely reactive chondrocytes for S-100 protein in the vicinity of foci of cartilaginous matrix mineralization and in the zone of provisional calcification in the epiphyseal growth plate.

The internal layer of osteogenic cells of the periosteum in long bones (Ham and Cromack, 1979) can potentially differentiate directly into osteoblasts and chondrocytes (Nakahara *et al.*, 1990). In the present study, we immunohistochemically localized those factors involved in the regulation of proliferation and differentiation of primitive mesenchymal cells during the course of bone formation in the bone fracture repair process.

MATERIALS AND METHODS

1. Experimental animals

Eight-week-old female Sprague-Dawley rats were purchased from Clea Japan (Tokyo, Japan). The animals were housed in plastic cages and provided with pellet food (MF: Oriental, Chiba, Japan) and water *ad libitum*. The experimental protocol was performed according to the rules and guidance of the Animal Care Committee at Fukui Medical University.

2. Fracture model

Rats were initially anesthetized with intraperitoneal pentobarbital (65 mg/kg body weight), then prepared for surgery by shaving and sterilization of the hind limbs. The knee was exposed through a medial patellar incision. A Kirschner wire (0.8 mm in diameter and 2.4 cm long) was introduced into the intramedullary canal and was carefully set so as not to protrude into the knee and ankle joint and prevent interference with the motion of the patella and foot. After closing the knee joint, closed fractures of the tibia were produced using a three-pointed bending clamp that reproducibly created a transverse non-comminuted mid-diaphyseal fracture.

3. Sampling and sectioning

Rats were sacrificed using an overdose of sodium pentobarbital. The fractured tibia was harvested at 1, 2, 3, 4, 5, 6, 7, 10 or 14 days after the fracture. Each specimen was fixed 10% buffered formaldehyde at 4°C for 48 hours, demineralized in 18% EDTA for 48 hours and embedded in paraffin. Serial 4 µm-thick sections were cut through the long axis of each tibia in the sagittal plane, and deparaffinized with xylene followed by ethanol.

4. Immunohistochemical staining

After washing with distilled water, intrinsic peroxidase was blocked with 0.3% H_2O_2 solution dissolved in absolute methanol at 20°C for 10 min and washed with phosphate buffered saline (PBS, pH 7.4). Sections were irradiated three times in a polypropylene slide-holder with a cap filled with PBS (pH 7.4), over a period of 5 min, using a microwave oven (500 w; ER-245; Toshiba, Tokyo). Sections were mounted with 2% skim milk (Yukijirushi, Sapporo, Japan) dissolved in PBS (pH 7.4) at 37°C for 30 min to block the background adsorption of antiserum. Then they were reacted with blocking solution (PBS containing carrier protein and 15 mM sodium azide; LSAB kit; Lot. No. 0075, Dako, Glostrup, Denmark) at 20°C for 10 min. This was followed by reaction with the following primary antibodies, respectively, at 4°C overnight: monoclonal anti-PCNA antiserum (mouse, PC10, NC-012, Lot 499, Novocastra Laboratory, Newcastle, UK, 50 µl dissolved 2.5 ml PBS at pH 7.4 added with 0.02% Triton X-100); polyclonal anti-TGF-β 1 antiserum (rabbit, V, SC-146, Lot B098, Santa Cruz Biotechnology, Santa Cruz, CA, 15 µl dissolved in 2 ml PBS pH 7.4); polyclonal anti-TGF-β 2 antiserum (rabbit, V, SC-90 Lot L187, Santa Cruz Biotechnology, 15 µl dissolved in 2 ml PBS pH 7.4); polyclonal anti-TGF-β 3 antiserum (rabbit, V, SC-82, Lot I237, Santa Cruz Biotechnology, 15 µl dissolved in 2 ml PBS, pH 7.4); polyclonal anti-TGF-β R1 antiserum (rabbit, V-22, SC-398, Lot I 097, Santa Cruz Biotechnology, 15 µl dissolved in 2 ml PBS, pH 7.4), polyclonal anti-TGF-β R2 antiserum (rabbit, L-21, SC-400, Lot K137, Santa Cruz Biotechnology, 15 µl dissolved in 2 ml PBS, pH 7.4), polyclonal anti-bFGF antiserum (rabbit, C-15, SC-121, Lot H227, Santa Cruz Biotechnology, 10 µl dissolved in 2 ml PBS, pH 7.4); monoclonal anti-bFGFR antiserum (mouse, VBS6, SC-276, Lot G088, Santa Cruz Biotechnology, 50 µl dissolved in 2.5 ml PBS, pH 7.4), polyclonal anti-PDGF antiserum (rabbit, N-30, SC-128, Lot G088, Santa Cruz Biotechnology, 10 µl dissolved in 2 ml PBS, pH 7.4), polyclonal anti-BMP2 antiserum (gout, A-20, SC-6267, Lot L087, Santa Cruz Biotechnology, 15 µl dissolved in 2 ml PBS, pH 7.4); and polyclonal anti-VEGF antiserum (rabbit, RB-222-P1, Lot LC222215, NeoMarkers, Union City, USA, 10 µl dissolved in 2 ml in PBS, pH 8.0). Sections were further reacted with LINK (biotinylated anti-mouse and anti-rabbit immunoglobulins in PBS, containing carrier protein and 15 mM sodium azide, LSAB, Dako) at 20°C for 60 min and rinsed with PBS, pH 7.4 and allowed to react with streptavidin solution (streptavidin conjugated to horseradish peroxidase in Tris-HCl buffer, LSAB kit, Dako) at 20°C for 30 min, and rinsed with PBS pH 7.4. In order to visualize the peroxidase color reaction, sections were incubated with DAB solution (DAB, CB090, Dojin, Kumamoto, Japan, dissolved in 100 ml of 0.05 M Tris-HCl buffer pH 7.4). Nuclear counterstaining was carried out with hematoxylin.

5. Alkaline phosphatase staining

Tissue were prepared for alkaline phosphatase staining according to the method described by Burstone and Keyes (1957) and Watanabe and Fishman (1963), and fixed in 10% buffered formaldehyde for 24 hours at 4°C, and further decalcified for 2-4 days in 0.5 M EDTA (0.05 M Tris-HCl buffer, pH 7.6) at 4°C. The tissue block was mounted in 20% Holt gum sucrose solution containing 30 g sucrose (Wako) and 1.0 g Arabic gum (Wako) dissolved in 100 ml of distilled water for 24 hr at 4°C and embedded with OCT compound (Lot. 0983084, Miles Inc., Elkhart, USA) followed by freezing. Frozen blocks were cut into 10 µm thick sections using cryostat (Cryocut 1800, Leica, Nusslosh, Germany). The frozen sections were allowed to thaw in a reaction solution containing 10 mg naphthol AS-BI phosphate acid sodium salt (Lot. CAN9061, Wako) and 10 mg Fast red violet LB salt (Lot. 07911PT, Aldrich) dissolved in 20 ml 0.05 M Tris-HCl buffer, pH 9.8 for 20 min at 2°C. After washing with water, nuclear counterstaining was carried out with hematoxylin, and mounted with glycerin (Lot. SDQ1161, Wako).

6. Quantification of cell proliferative activity

In order to determine the percentage PCNA (proliferating cell nuclear antigen)-positive cells (PCNA

index), we counted the number of cells with brown DAB reaction product among at least 1,000 cells. This was performed for each cell lineage, primitive mesenchymal cells at the fracture edge, periosteal mesenchymal cells, osteoblasts, chondroblasts and chondrocytes at the fracture edges, and vascular endothelial cells at both sites. Counting was performed under a light microscope (×40 objective and ×10 eye piece, Olympus, Tokyo).

7. Evaluation of extent of immunohistochemical staining

We also determined the percentage of cells positive for each immunohistochemical stain among at least 1,000 cells of each cell lineage, and the extent of positivity was classified into four groups, negative (-), <10%(+), 10-50% (++), and >50% (+++).

RESULTS

1. Histology of the fracture repair process

At 1-day after experimentally-induced fracture, numerous mesenchymal cells, and inflammatory cells including macrophages, were mobilized at the fracture edges with hematoma formation. At 2-days after fracture, the number of undifferentiated mesenchymal cells increased in the periosteum neighboring the fracture edges, and a small number of osteoblasts positively stained with alkaline phosphatase reaction began to appear. At 3-days after fracture, morphologically identified chondroblasts and chondrocytes began to appear at the fracture edges, and hypertrophic chondrocytes appeared first at 5-days after fracture. At 3-days after fracture, primitive callus was also noted to appear in the periosteum with marrow spaces lined by many osteoblasts and osteoclasts, while vascular ingrowth became apparent. At 5-days after fracture, vascular ingrowth with branching begun to be apparent in cartilaginous tissue formed at the fracture edges. At 7-days after fracture, bone began to form from the cartilage at fracture edges by a process that resembled that of bone formation in the growth plate. The enchondral ossification established a bridge between both fracture edges and periosteal membranous ossification encompassed the fracture site like a sheath at 14-days after fracture.

2. Changes in PCNA index in different cell lineages during fracture repair

At control conditions (prior to the fracture), bone marrow spaces contained hematopoietic cells but no PCNA positive cells. A small number of PCNA positive cells began to appear among mesenchymal cells including inflammatory cells, which mobilized at the fracture edges at 1-day after fracture, and the PCNA index reached its peak level at 2-days (Fig. 1a), followed by rapid decrease up to 6-days (Fig. 5). PCNA positive cells also appeared among mesenchymal cells and primitive osteoblasts in the periosteum at 2-days and the peak PCNA index was noted at 3-days (Fig. 1b), followed by a steep fall toward 7-days (Fig. 5). This periosteal cell proliferation and cytodifferentiation of mesenchymal cells to osteoblasts were apparent in the neighboring areas of the fracture. PCNA positivity began to appear in primitive chondroblasts (Fig. 1c) and chondrocytes (Fig. 1d) derived from mesenchymal cells at the fracture edges at 3-days after fracture, and the PCNA index reached its peak value at 4-days, followed by a gradual decrease to 10-days (Fig. 5). The peak values of PCNA indexes for ingrowing vascular endothelial cells in the periosteal membranous ossification and enchondral ossification at the fracture edges were noted at 4-days and 5-days after fracture (Fig. 6).

3. Immunohistochemical staining for various growth factors

Results of immunohistochemical studies are summarized in Table I, and shown in Fig. 1 including PCNA staining. All cell lineages, periosteal mesenchymal cells, osteoblasts, chondroblasts and hypertrophic chondrocytes at the fracture edges showed positive immunohistochemical staining for PDGF, TGF-β 1,2 (Fig. 2a and 2b), 3 and their receptors (Fig. 2c and 2d), although the percent of positive cells varied from one cell type to another. Although periosteal mesenchymal cells, osteoblasts, and chondroblasts were positive for BMP, PDGF, bFGF (Fig. 3a, b, and c), and its receptor, hypertrophic chondrocytes were negative for them (Fig. 3d).

VEGF was only positive in chondroblasts and hypertrophic chondrocytes at the fracture edges (Fig. 4a). S-100 protein was also only positive in these cells (Fig. 4b).

Fig. 1 - Immunohistochemical staining of PCNA (**a-d**)(×170). **a:** 2-days after fracture, **b:** 3-days after fracture, **c:** 4-days after fracture, **d:** 7-days after fracture.
Fig. 2 - Immunohistochemical staining of TGFβ2 (**a-b**) and TGFβRII (**c-d**)(×170). **a-c:** 7-days after fracture, **d:** 5-days after fracture.

Fig. 3 - Immunohistochemical staining of bFGF (**a-d**)(×170). **a:** 3-days after fracture, b**:** 4-days after fracture, c: 5-days after fracture, d: 7-days after fracture.
Fig. 4 - Immunohistochemical staining of VEGF (**a**) and S100 (**b**) (×340). **a:** 3-days after fracture, **b:** 4-days after fracture.

DISCUSSION

The major finding of the present study is the completion of the fracture repair process through two different processes, cell proliferation and differentiation. The first process involves bone formation from the cartilage at the fracture edges, which resembles the process of bone formation in the growth plate of long bone, lasting for 7 days before fusion starting at 7-days after fracture, and the process of periosteal intramembranous ossification commencing 3-days after fracture. The latter, on both sides of the fracture, also fused with each other and encompassed the fracture site like an outer sheath, also around 14-days after fracture.

Joyce *et al.* (1990) proposed four distinct histologic stages of fracture healing, based on cellular features, extracellular matrix and time of appearance of tissue components. These included the immediate injury response (stage I, 2-days after fracture), intramembranous bone formation (stage II, 4-days), chondrogenesis (stage III, 10-days) and enchondral ossification (stage IV, 15-days). They illustrated that the enchondral ossification occurs at the fracture sites of the periosteal intramembranous ossification but not at the bone fracture edges, filling the fracture portion like a wedge from outside. The time course of cytodifferentiation reported by these investigators is delayed for 1-8 days for all cell lineages in comparison to those found in the present study. In both the above and present studies, the method developed by Bonnarens and Einhorn (1984) was used to produce a standard closed fracture in laboratory animal bone. However, the femur was used in the above study while we used the tibia in order to minimize the movement of the fractured bone with the aid of the intact fibula. As pointed already by Bonnarens and Einhorn (1984), fractures vary with respect to their locations, mechanism of injury, and associated soft tissue trauma. These variables must be carefully controlled when designing experiments for the study of fracture repair. Thus, a larger weight loading and movability may be superimposed on the femur possibly with larger soft tissue damages than tibia. These differences in experimental design might explain the different results in the two studies.

Another interesting finding in our study is the sequential appearance of cell proliferation peaks in different locations and different cell lineages as shown by PCNA immunohistochemistry (Fig. 5).

Table I

Summary of the results of immunohistochemical stainings of growth factors and their receptors in each cell lineage. The extents of positivity are classified into four groups; no positive cells (-), less than 10% of positive cells (+), 10% to 50% of positive cells (++), and more than 50% of positive cells (+++).

Tissue	BMP2	PDGF	TGFβ1	TGFβ2	TGFβ3	TGFβ R1	TGFβ R2	bFGF	FGFR	VEGF	S100
Intramembranous ossification											
Mesenchymal cell	+	+	+	++	++	+	+	++	+	-	-
Osteoblast	+	++	+	++	++	+	++	++	+	-	-
Endochondral ossification											
Mesenchymal cell	+	+	+	++	++	+	+	+	+	-	-
Chondroblast	+	++	+	+++	+++	++	++	+++	++	+	+++
Mature chondrocyte	-	++	+	+++	+++	+++	+++	-	-	+++	+++

BMP2 bone morphogenetic protein; PDGF platelet derived growth factor; TGFβ transforming growth factor β; bFGF basic fibroblast growth factor; FGFR fibroblast growth factor receptor; VEGF vascular endothelial growth factor

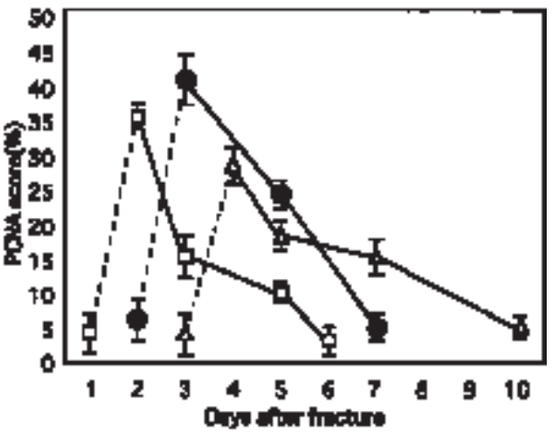

Fig. 5 - Changes of PCNA scores in each cell lineage during the experimental time course. Open square, mesenchymal cell and macrophage; closed circle, periosteal cell and osteoblast; open triangle, chondrocyte and chondroblast.

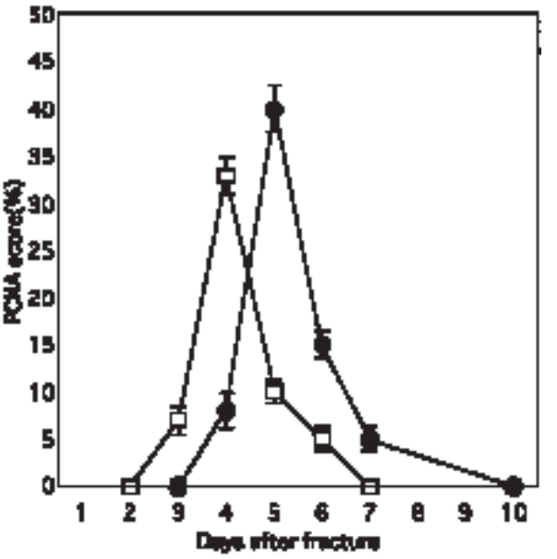

Fig. 6 - Changes of PCNA scores in vascular endothelial cells during the experimental time course. Open square, intramembranous ossification; closed circle, endochondral ossification.

The earliest event in the healing process is the activation of proliferative activity of primitive mesenchymal and inflammatory cells including macrophages at the fracture site, followed by those of internal osteogenic layer cells in the periosteum adjacent to the fracture, and chondroblasts at the fracture edges (Fig. 5). These results clearly indicate that various growth factors that stimulate cell proliferation are first secreted at the fracture site and that a such process spreads in a waveform pattern to adjacent tissues. In this process, BMP2 stored in bone matrix, PDGF, TGF-β, and bFGF accumulated in macrophages and platelets are immediately released into the fracture site and induce the proliferation of primitive mesenchymal cells as well as their differentiation to osteoblasts and chondroblasts. Interestingly, differentiation of primitive mesenchymal cells to chondroblasts seems to take longer time than that to osteoblasts (Fig. 5), although the mechanism of this differential response remains unknown at present. As shown in Table I, BMP2, bFGF and their receptor were immunohistochemically undetectable on hypertrophic chondrocytes, although they were positive on primitive mesenchymal cells, osteoblasts and chondroblasts, indicating that the cytodifferentiation of chondroblasts to hypertrophic chondrocytes occurs without the need for these growth factors. This also indicates that neovascularization in the intramembranous ossification requires bFGF as an angiogenic factor, but that in enchondral ossification does not depend on this factor. In accordance with this interpretation, VEGF stimulated-endothelial cell replication was only positive immunohistochemically for chondroblasts and hypertrophic chondrocytes at the fracture edges (Table I). S-100 protein containing binding sites specific for Ca^{2+} was also positive only in these cells (Table I). These results clearly show that the process of enchondral ossification at fracture edges is dependent on neovascularization induced by VEGF and calcium binding by S-100 protein. These interpretations seems to be also supported by the finding that the peaks of vascular endothelial cell proliferation in periosteal intramembranous ossification (4-days) and enchondral ossification at the fracture edges (5-days) was delayed one day after the corresponding peaks of periosteal osteoblasts positive for bFGF at 3-days and hypertrophic chondrocytes at the fracture edges at 5-days after fracture, respectively (Fig. 6).

Cells positive for TGF-β and bFGF were also positive for their receptors (Table I), suggesting that the growth factors were initially released from the fractured bone, and macrophages and platelets induced further production of these factors in differentiated osteoblasts and chondroblasts, thus

switching the growth regulation system from paracrine to autocrine system.

In summary, the closed fracture model prepared according to the method of Bonnarens and Einhorn (1984) is a good model of fracture repair, and the tibia is a better site than femur for obtaining reproducible results. Our results demonstrated the presence of two different steps of ossification, periosteal intramembranous ossification and enchondral ossification at the fracture edges, taking place during the course of fracture repair. These processes are enhanced by different types of growth factors for cell proliferation and differentiation, together with neovascularization and mineralization.

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