Regulation of Fracture Repair by Growth Factors (43410A)

MARK E. BOLANDER[1]
Department of Orthopaedics, Mayo Clinic, Rochester, Minnesota 55905

Fracture repair is unique when compared with healing after soft tissue injury; although both healing responses involve the formation of new tissue at the site of injury, soft tissues heal by replacing the injured tissue with a fibrous scar, whereas bone heals by regeneration of the normal osseous anatomy. The cellular events responsible for the repair of a bone injury include, in addition to proliferation of fibroblasts and the influx of inflammatory cells found in soft tissue wounds, the differentiation of chondrocytes and osteoblasts from progenitor cells and the formation of bone by endochondral ossification (1–3). Despite this added complexity, the cellular events in fracture repair proceed in an orderly and reproducible fashion, leading to a well-defined pattern of repair when viewed in histological sections (4, 5).

Four Stages of Fracture Repair

The histologic progression of fracture repair can easily be divided into four distinct stages, each characterized by different cellular features and an extracellular matrix (Fig. 1).

Stage 1: Immediate Injury Response. Immediately after fracture, a hematoma forms at the fracture site that extends along the cortex above the periosteum and into the overlying soft tissue and muscle. Undifferentiated cells adjacent to the hematoma in the periosteum, muscle, and the surrounding tissues proliferate. These tissues are invaded by macrophages and other inflammatory cells as the fibrous clot is organized into granulation tissue. These processes result in the formation of a reparative granuloma at the site of fracture that is called an external callus.

Stage 2: Intramembranous Ossification. New bone matrix is synthesized by osteoblasts located adjacent to the fracture site and between the proliferating periosteal cells and the underlying cortex. Bone formation in the periosteum occurs by differentiation of osteoblasts from precursor cells without a cartilaginous intermediate. The region of bone formation in the external callus is called the hard callus.

Stage 3: Chondrogenesis. Mesenchymal or undifferentiated cells also are seen in granulation tissue overlying the fracture site. As healing progresses and intramembranous bone matures, undifferentiated cells immediately adjacent to areas of intramembranous bone become larger and synthesize an avascular basophilic matrix. This region is similar in appearance to the cartilaginous matrix in the proliferating zone of the growth plate. The cartilaginous region enlarges as more mesenchymal cells develop the histological features of chondrocytes, and continues until all fibrous tissue in this soft callus is replaced by cartilage. The fibrous and cartilaginous region of the external callus is called the soft callus.

Stage 4: Endochondral Ossification. Bone forms from cartilage in the soft callus by a process that appears similar to bone formation in the growth plate. Chondrocytes adjacent to the subperiosteal bone hypertrophy, the cartilage extracellular matrix calcifies, and capillaries from adjacent bone invade the calcified cartilage. Osteoblasts follow capillary ingrowth and synthesize osteoid on the calcified cartilage, forming primary spongiosa with the distinctive "mixed spicule" that contains both bone and cartilage. This process continues until all cartilage in the soft callus is replaced by bone, "bridging" the fracture gap. when bone bridging has occurred, mechanical stability is restored, and remodeling of new bone and underlying bone cortex restores the normal bone architecture.

Regulation of Fracture Repair

While each of the four stages of fracture repair has distinct histological features, they share several underlying cellular events that are subject to regulation. These events include cell proliferation and differentiation, chemotaxis, and the synthesis of extracellular matrix. The repair of fractures is believed to be regulated by both systemic and local factors. Systemic factors that affect fracture repair are well characterized in the literature, and include endocrine, metabolic, and genetic

[1] To whom requests for reprints should be addressed at Department of Orthopaedics, Med Science Room 3–69, Mayo Clinic, 200 First Street SW, Rochester, MI 55905.
P.S.E.B.M. 1992, Vol 200: 165–170

0037-9727/92/2002-0165$3.00/0

factors and drug treatment. Local factors are appreciated as important in fracture repair, but are less well characterized.

Local regulators of fracture repair could be secreted by both inflammatory and noninflammatory cells. Current investigations indicate that macrophages and other inflammatory cells at sites of injury in nonskeletal tissues secrete cytokines and growth factors that are critical regulators of healing. The presence of inflammatory cells in the fracture callus suggests that macrophages in the callus also secrete cytokines and growth factors to regulate the initial stages of fracture repair. The literature on growth factors' effects on cells *in vitro* contains many examples demonstrating growth factors' regulation of musculoskeletal cell function, including stimulation of proliferation by chondrocytes, osteoblasts, and periosteal cells, initiation of chondrocyte differentiation and the expression of Type II procollagen in the periosteum, and modulation of extracellular matrix synthesis by chondrocytes and osteoblasts. As similar cellular events occur in the fracture callus, these studies suggest that growth factors also act as regulators of cell differentiation and matrix synthesis in the later stages of fracture repair.

Growth factors synthesized by macrophages include transforming growth factor-β1 (TGF-β1), the acidic and basic fibroblast growth factors (aFGF and bFGF), as well as interleukins and other cytokines. Platelet degranulation during hematoma formation is also a significant source of growth factors, including TGF-β1 and platelet-derived growth factor (PDGF). Other sources of growth factors include osteoblasts (FGF, TGF-β, and PDGF) and the bone matrix itself, which contains high concentrations of FGF and TGF-β. The number of growth factors likely to have a significant role in the regulation of wound repair, osteogenesis, and chondrogenesis is increasing. A partial list of growth factors that affect healing is presented in Table I.

Growth factors could be synthesized by osteoblasts, macrophages, or chondrocytes within the fracture cal-

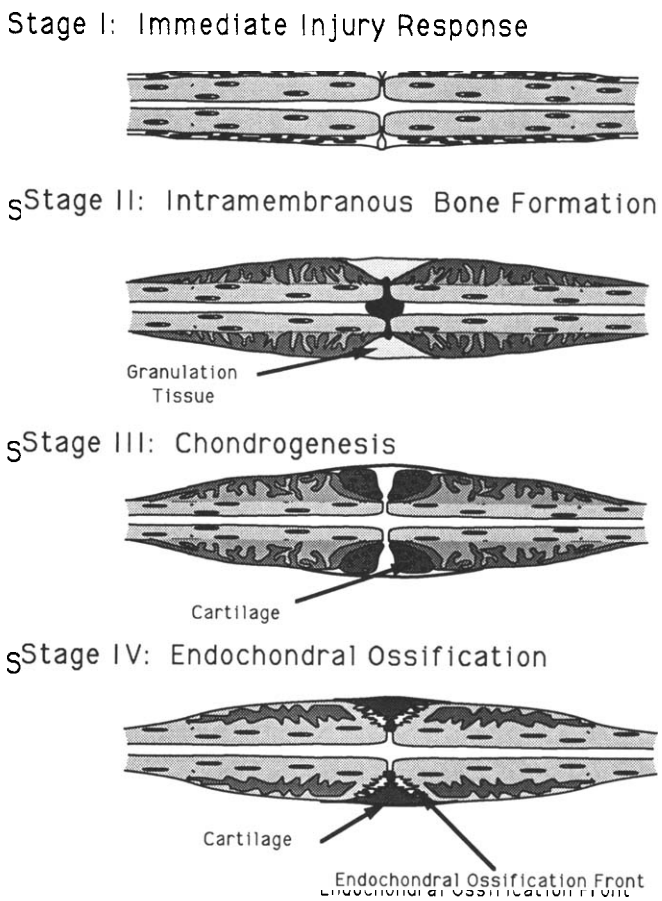

Figure 1. The four cellular stages of fracture repair: the immediate injury response (Stage I), intramembranous ossification (Stage II), chondrogenesis (Stage III), and endochondral ossification (Stage IV). Arrows indicate areas of granulation tissue, cartilage, and the endochondral ossification front.

Table I. Growth Factors and Fracture Healing

Transforming Growth Factor-β	
Source:	Platelets, inflammatory cells (monocytes, macrophages), osteoblasts, chondrocytes
Matrix:	Bone is the most abundant source of TGF-β in the body
Responding cells:	Most cells have TGF-β receptors
Unique characteristics:	Inactive precursor peptide, most potent chemoattactant identified for macrophages, promotes angiogesis
Fibroblast Growth Factors (aFGF, bFGF)	
Source:	Inflammatory cells, osteoblasts, chondrocytes
Matrix:	Active matrix bound form (HSPG),[a] bone is a FGF reservoir
Responding cells:	Most cells of mesoderm or neuroectoderm origin
Unique characteristics:	Stimulates neovascularization, evidence for an autocrine, intracellular, function; stimulates Type IV collagenase
Platelet-Derived Growth Factor (PDGF-AA, AB, BB)[b]	
Source:	Platelets, monocytes, activated tissue macrophages, endothelial cells
Matrix:	Interactions unknown
Responding cells:	Most cells of mesoderm origin
Unique characteristics:	Receptor tyrosine kinase activity, well-described receptor subunit interactions

[a] FGF binds to heparin sulfate proteoglycan (HSPG).
[b] Two PDGF genes combine to form three isoforms: AA, A chain homodimer; AB, A-B heterodimer; and BB, B chain homodimer.

lus. Alternatively, growth factors may be delivered to the fracture callus by the bloodstream, or, more likely, they could be released by platelets into the fracture hematoma (6). Growth factors may regulate fracture repair by paracrine or autocrine pathways (7), and can exert a broad spectrum of activities. Consequently, determination of the precise location of different growth factors is important for elucidating its ultimate role. Although an increase in growth factor gene expression preceding a histological or cellular event suggests a role for growth factor regulation, the evidence is only correlative and does not provide direct data on the role of the growth factor. Direct evidence for growth factor regulation can be obtained by adding exogenous factors to specific stages of fracture healing *in vivo* and *in vitro*, or by testing growth factors in models of different aspects of the fracture repair response.

Synthesis and Localization of Growth Factors in the Fracture Callus

The presence of a growth factor within the fracture callus is obviously a prerequisite for the growth factor to exert regulatory actions during fracture healing. Experimentally, growth factors are localized to cells and regions within the callus by immunostaining of the fracture callus and monospecific antibodies. Gene expression for different growth factors is detected by Northern analysis if expression occurs at high levels, or by amplification techniques (polymerase chain reaction amplification of RNA after reverse transcription) if the growth factor gene is expressed at low levels or by a small number of cells.

Growth Factor Gene Expression during Fracture Healing. To determine whether growth factors are expressed by cells within the fracture callus, and, if so, at which stages of the fracture-healing process, RNA extracted from fracture calluses was evaluated by Northern hybridization for TGF-β1 and PDGF gene expression (8) and by reverse transcription followed by polymerase chain reaction amplification to detect acidic and basic FGF gene expression (9). These studies show that the levels of growth factor gene expression vary with the stage of fracture repair (Table II). Expression of the TGF-β1 gene is high during chondrogenesis (Stage III) and endochondral ossification (Stage IV), but lower during intramembranous bone formation (Stage II). No expression is detected during the immediate injury response (Stage I). Acidic FGF gene expression also varies with the stage of repair, and maximal expression is seen during chondrogenesis (Stage III). Constant levels of bFGF and PDGF B expression are detected during all stages of fracture repair.

These observations suggest that different growth factors are expressed in the callus during different cellular events. The absence of TGF-β1 gene expression in Stage I indicates that little, if any, of this growth factor is synthesized early in fracture repair. High levels of expression of TGF-β1 and aFGF in Stage III suggest that chondrocytes synthesize high levels of these growth factors. Lower levels of expression for other growth factors suggest that these growth factors are also synthesized by cells in the callus, but these analyses do not detect changes in the relative levels of gene expression.

Table II. Growth Factor Gene Expression during Fracture Healing

	Fracture healing stage[a]			
	I	II	III	IV
TGF-β1	0	++	++++	+++
aFGF	+	+	++	+
bFGF	+	+	+	+
PDGF-B (c-sis)	0	++	++	++
PDGF-A	ND	ND	ND	ND

[a] Fracture healing stages defined as: I, initial injury response; II, intramembranous ossification; III, chondrogenesis; IV, endochondral ossification. ND, not determined; 0, not detectable by Northern blotting; +, detectable by reverse transcription followed by PCR amplification; ++, less than 50% maximal expression on Northern blotting; +++, greater than 50% maximal expression on Northern blotting; ++++, maximal expression on Northern blotting.

Growth Factor Immunolocalization during Fracture Healing. Growth factors in the fracture callus can be localized to specific cell types by immunostaining with monospecific antibodies. Using the histological stages of fracture healing as a reference, distinct immunostaining patterns are seen for TGF-β, aFGF, and bFGF.

Extracellular TGF-β1 is localized to the hematoma as early as 24 hr after fracture, both at the fracture site and along the periosteum (Fig. 2). In Stage I, immunostaining of the hematoma and periosteum persists for several days after fracture and precisely defines the region of periosteal proliferation and intramembranous bone formation (Fig. 2b). In Stage II, intramembranous bone adjacent to the fracture site demonstrates intercellular staining of the osteoblasts in the bone spicules for TGF-β1 (Fig. 2, c, e, and f). In Stage III, mesenchymal cells and immature chondrocytes stain intensely for intercellular TGF-β1 (Fig. 2i), while the surrounding hematoma and matrix demonstrate extracellular TGF-β1 staining (Fig. 2h). The cartilaginous matrix surrounding mature chondrocytes demonstrates little or no TGF-β1 staining (not shown), whereas the matrix that surrounds hypertrophic chondrocytes in Stage IV stains strongly for extracellular TGF-β1 (Fig. 2k). Also in Stage IV, ossified matrix on the bone side of the ossification front does not stain for TGF-β1, whereas the osteoblasts that line this bone matrix exhibit intercellular TGF-β1 staining (Fig. 2l).

Similar studies have evaluated basic FGF in the fracture callus (Table III). During Stage I, bFGF stains macrophages in the granulation tissue in the developing fracture callus. Anti-bFGF antibodies first stain osteoblasts during the intramembranous bone formation

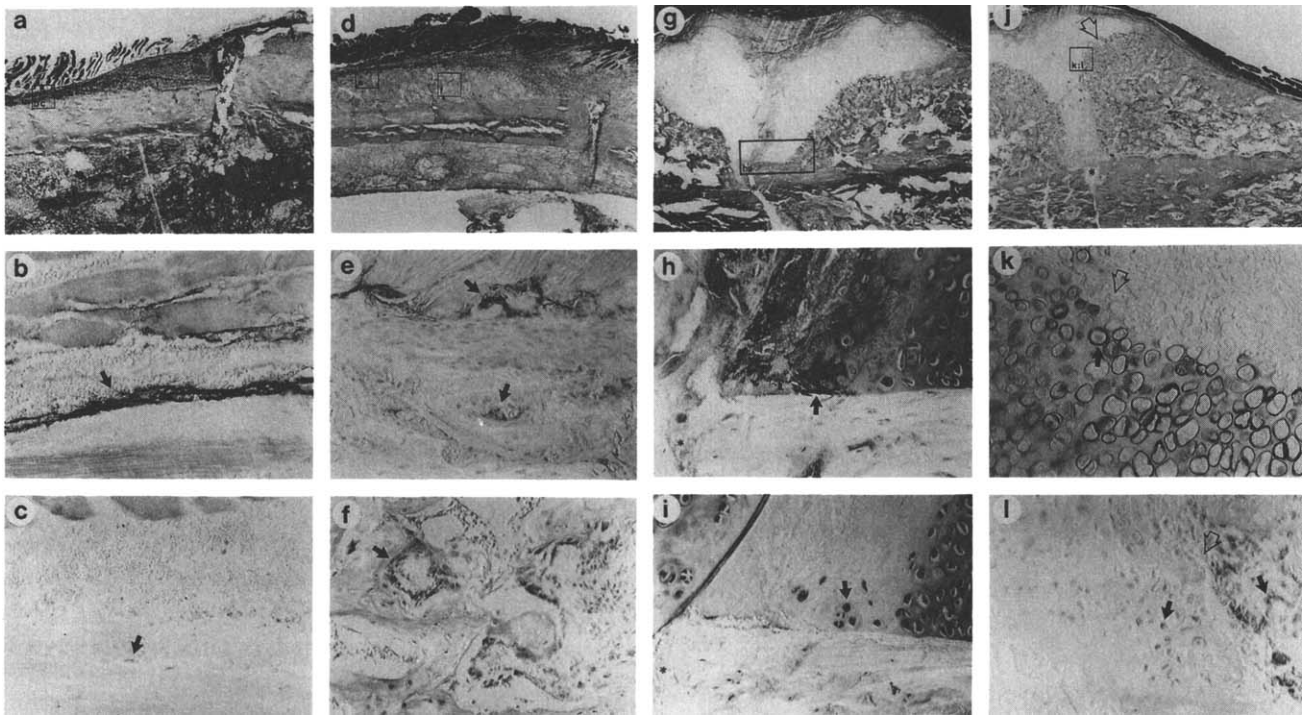

Figure 2. Immunostaining of the fracture callus with antibodies for TGF-β1 (For details, see text.).

Table III. Immunolocalization of Growth Factors during Fracture Healing

	Fracture healing stage[a]			
	I	II	III	IV
TGF-β1	Fibrous periosteum	Osteoblasts	Chondrocytes, cartilage matrix	Hypertrophic chondrocytes, calcified cartilage matrix
aFGF	Macrophages, periosteal cells	Osteoblasts	Immature chondrocytes	Osteoblasts
bFGF	Macrophages	Osteoblasts, bone matrix	Chondrocytes	Hypertrophic chondrocytes, osteoblasts, bone matrix

[a] Fracture healing stages defined as: I, initial injury response; II, intramembranous ossification; III, chondrogenesis; IV, endochondral ossification.

(Stage II). Immature chondrocytes in Stage III show staining for bFGF in the nucleus, whereas mature chondrocytes stain more intensely in the peripheral portion of their cytoplasm. Osteoblasts continue to demonstrate intercellular bFGF staining throughout endochondral ossification (Stage IV). Additionally, there is bFGF staining of the osteoblast pericellular matrix, especially at the endochondral ossification front, suggesting the extracellular accumulation of bFGF in the bone matrix.

In contrast to the intracellular and extracellular staining for both TGF-β and bFGF, immunohistological staining with aFGF antibodies shows exclusively intercellular staining throughout the fracture-healing process. Macrophages in Stage I stain intensely for aFGF. The cytoplasm of preosteoblasts in regions of intramembranous and endochondral ossification (Stages II and IV) stain lightly for aFGF. Chondrocytes demonstrate a differential aFGF staining pattern, with immature chondrocytes staining more intensely than mature or hypertrophic chondrocytes.

Correlation of Growth Factor Expression with Histology. Once a growth factor has been localized within the fracture callus, correlating the expression and location of the growth factor with changes in the histology of the callus may suggest a role for growth factor regulation. This analysis implies that TGF-β1 detected in the fibrous periosteum during Stage I is not the result of synthesis by periosteal cells, but results from binding of growth factor synthesized elsewhere. Significant concentrations of TGF-β1 are released from platelets into the hematoma at the time of injury, suggesting that platelets are the source of TGF-β1 seen in the periosteum. The accumulation of TGF-β1 in the periosteum and the clear association between TGF-β1 staining and the extent of callus formation suggest that TGF-β1 may stimulate proliferation during initial cal-

lus formation. TGF-β1 in chondrocytes during Stage III and in hypertrophic chondrocytes of the callus during Stage IV are probably synthesized by these cells, as high levels of TGF-β1 gene expression are detected in Stage III. Accumulation of TGF-β1 in the hypertrophic matrix, but not in the matrix around mature chondrocytes, suggests that chondrocytes transport this growth factor from intracellular sources into the extracellular matrix. TGF-β1 in this matrix may have a role in regulating cartilage matrix calcification and endochondral ossification.

Acidic FGF is expressed at lower levels, but increased expression in Stage III suggests a role in chondrogenesis. Basic FGF is also expressed, but at constant levels; consequently, this analysis is not informative about the possible effect of this growth factor on cell activity.

Growth Factors in Models of Fracture Repair

This evidence of growth factor function is only suggestive, however, and does not provide any direct data on the role of the different growth factors. Direct evidence for growth factor regulation can be obtained by adding exogenous growth factors to specific stages of fracture healing *in vivo*. It can be technically difficult to add exogenous growth factors to healing fractures *in vivo* and generate results that can be interpreted. Therefore, growth factors also have been studied in models of different stages of fracture callus formation and development.

To evaluate the significance of increased expression of aFGF in Stage III, aFGF was injected into the soft callus during early chondrogenesis. Analysis of the repair process after injection demonstrated increased cartilage in the callus, but a delay in the initiation of endochondral ossification. These observations suggest that aFGF injection stimulated chondrocyte proliferation, but delayed maturation. No effects were seen on other cell types in the callus, and bone formation was unaffected (10).

Platelets are the first source of growth factors during the initiation of fracture repair. The proliferation and differentiation of periosteal mesenchymal cells in Stage I is likely to be initiated or modulated by growth factors released from platelets that degranulate during hematoma formation. This aspect of fracture healing can be modeled in experimental animals by injecting growth factors found in platelets into the subperiosteal tissue of uninjured femurs.

Subperiosteal injections of TGF-β into the nonfractured rat femur result in mesenchymal cell proliferation and the subsequent initiation of chondrogenesis and intramembranous bone formation. Mesenchymal cell proliferation in the inner cambial layer of the periosteum is seen after injection of 200 ng of TGF-β1 (Fig. 3). After 4 days, chondrocytes are identified above

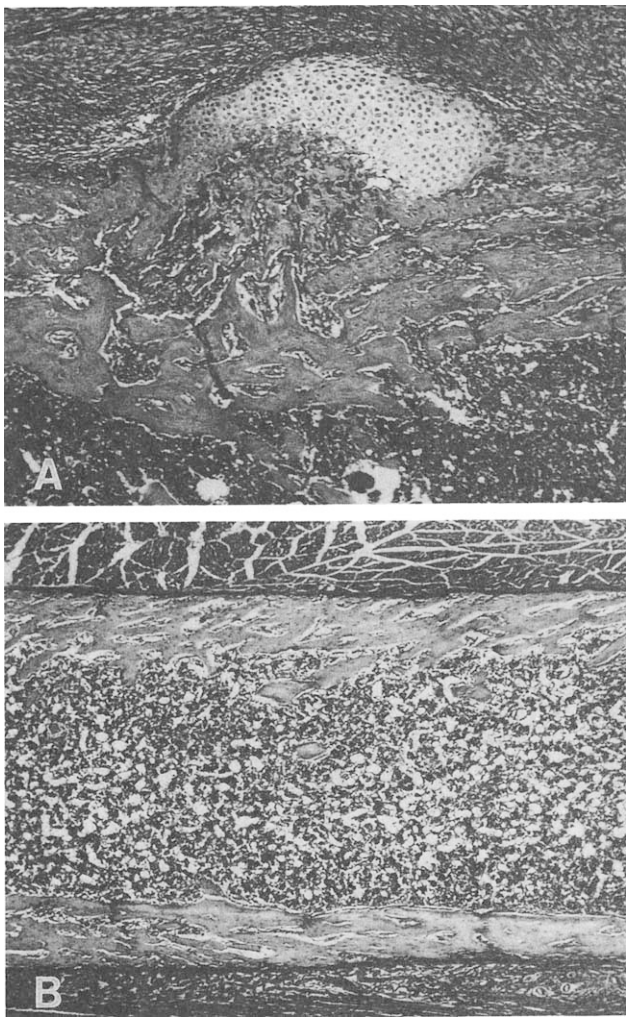

Figure 3. Formation of cartilage and intramembranous bone after four injections of 200 ng TGF-β1 into nonfractured subperiosteal tissue in the femur.

the cortex at the site of injection. The size of the cartilaginous mass increased severalfold. As the cartilage mass enlarges, hypertrophic chondrocytes develop near the underlying cortex, while smaller, proliferating chondrocytes are seen near the periosteum. New bone forms in the subperiosteal region lateral to cartilage by intramembranous ossification. After stopping TGF-β injections, the cartilaginous mass is replaced with bone by endochondral ossification. This bone undergoes active remodeling by osteoclasts, finally resulting in the thickening of the cortical bone.

Subperiosteal injection of PDGF results in a different response than that seen after injection of TGF-β1. PDGF also stimulates mesenchymal cell proliferation in the cambial layer of periosteum, and a bone mass forms in PDGF-injected femurs. No cartilage is seen in this mass, however, and it appears that new bone formation in PDGF-injected femurs is the result of intramembranous ossification. This bone also undergoes

active remodeling and results in the thickening of the cortex.

Summary

Fractured bones heal by a cascade of cellular events in which mesenchymal cells respond to unknown regulators by proliferating, differentiating, and synthesizing extracellular matrix. Current concepts suggest that growth factors may regulate different steps in this cascade (10). Recent studies suggest regulatory roles for PDGF, aFGF, bFGF, and TGF-β in the initiation and the development of the fracture callus. Fracture healing begins immediately following injury, when growth factors, including TGF-β1 and PDGF, are released into the fracture hematoma by platelets and inflammatory cells. TGF-β1 and FGF are synthesized by osteoblasts and chondrocytes throughout the healing process. TGF-β1 and PDGF appear to have an influence on the initiation of fracture repair and the formation of cartilage and intramembranous bone in the initiation of callus formation. Acidic FGF is synthesized by chondrocytes, chondrocyte precursors, and macrophages. It appears to stimulate the proliferation of immature chondrocytes or precursors, and indirectly regulates chondrocyte maturation and the expression of the cartilage matrix. Presumably, growth factors in the callus at later times regulate additional steps in repair of the bone after fracture.

These studies suggest that growth factors are central regulators of cellular proliferation, differentiation, and extracellular matrix synthesis during fracture repair. Abnormal growth factor expression has been implicated as causing impaired or abnormal healing in other tissues, suggesting that altered growth factor expression also may be responsible for abnormal or delayed fracture repair. As a complete understanding of fracture-healing regulation evolves, we expect new insights into the etiology of abnormal or delayed fracture healing, and possibly new therapies for these difficult clinical problems.

This work was partially supported by grants from the American Academy of Orthopaedic Surgeons and the Orthopaedic Research and Education Foundation.

1. Ham AW. A histological study of the early phase of bone repair. J Bone Joint Surg **30**:827–844, 1930.
2. Simmons DJ. Fracture healing perspectives. Clin Orthop Rel Res **200**:100–113, 1985.
3. Urist MR, Wallace TH, Adams T. The function of fibrocartilaginous fracture callus, observations on transplants labeled with tritiated thymidine. J Bone Joint Surg **478**:304–318, 1966.
4. Cruess RL, Dumont J. Fracture healing. Can J Surg **18**:403–413, 1975.
5. Nemeth GG, Heydemann A, Jingushi S, Kanan SM, Macey LR, Bolander ME. Expression of tissue specific and possible regulatory genes in a rat model of fracture healing. Am Fed Clin Res Abs, May 1988.
6. Assoian RK, Sporn MB. Type-β transforming growth factor in human platelets: Release during platelet degranulation and action on vascular smooth muscle cells. J Cell Biol **102**:1217–1223, 1986.
7. Sporn MB, Todaro GH. Autocrine secretion and malignant transformation of cells. N Engl J Med **303**:878–880, 1980.
8. Joyce ME, Jingushi S, Bolander ME. Transforming growth factor-beta in the regulation of fracture repair. Orthop Clin North Am **21**:199–209, 1990.
9. Scully SP, Joyce ME, Abidi N, Bolander ME. The use of polymerase chain reaction generated nucleotide sequences as probes for hybridization. Mol Cell Probes **4**:485–495, 1990.
10. Jingushi S, Heydemann A, Kana SK, Macey LR, Bolander ME. Acidic fibroblast growth factor (aFGF) injection stimulates cartilage enlargement and inhibits cartilage gene expression in a rat fracture model. J Orthop Res **8**:364–371, 1990.