

Developmental aspects of fracture healing and the use of pharmacological agents to alter healing

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Abstract

Fracture healing is a specialized postnatal repair process that recapitulates many aspects of embryological skeletal development. While many of the molecular mechanisms that control cellular differentiation and growth during embryogenesis recur during fracture healing, these processes take place in a postnatal environment that is unique and distinct from those which exist during embryogenesis. A number of the central biological processes that are believed to be crucial in the embryonic differentiation and growth of skeletal tissues and play a functional role in fracture healing are reviewed. The functional modification of these various developmental processes of fracture healing is discussed in the context of how different pharmacological agents might alter fracture healing.

Keywords: Fracture, Bone Morphogenesis, 3-D Reconstruction, Inflammation

Introduction

Our present understanding of the basic mechanisms of skeletal repair suggests that it is a multi-faceted process involving complex interactions between the immune, hematopoietic, and vascular systems, which all interact with the underlying mesenchymal cell population that contribute to the induction growth and/or maintenance of skeletogenic cell differentiation. Moreover, as bone is one of the few tissues in the body that undergoes a true regenerative response, many of the mechanisms involved in skeletal repair appear to recapitulate embryologic developmental events^{1,2,3}. A greater understanding of these events could lead to new strategies for optimizing skeletal repair and restoring segments of the skeleton, which have been lost because of injury or surgical resection. Studies to date have shown how many metabolic conditions, aging, and a variety of pharmacological agents affect skeletal repair. More recently, the molecular regulation of fracture healing has been partially elucidated and this has allowed investigators to postu-

late how growth factors, gene therapy, and other biological strategies may be used for therapeutic purposes.

Anatomy of fracture healing

The end result of the developmental processes that contribute to fracture repair is the regeneration of both the original geometry and biomechanical competency of the damaged bone. Like embryological development and skeletal growth, fracture repair involves the definition of specific morphogenetic fields and is thus dependent on instructive interactions between various proximate tissues. A histological overview of fracture healing (Figure 1) shows the overall spatial relationships, tissue development and the morphogenetic fields that establish tissue regeneration during fracture healing. Such analysis shows that two discrete centers of cartilage tissue are formed in a symmetric manner with respect to the fracture line that taper proximally and distally along the cortices of the bone. Concurrently, intramembranous bone formation is initiated proximally and distally in the periosteum and taper inward towards the fracture line deep to the ring of cartilage tissue. Three-dimensional reconstruction of serial histological sections from both murine and rat fracture callus tissues (Figure 2) surprisingly showed that callus tissues actually form in an asymmetric manner with the majority of the cartilaginous callus seen on the distal lateral surface for simple mid-diaphyseal transverse femur fractures. It is further interesting to note that

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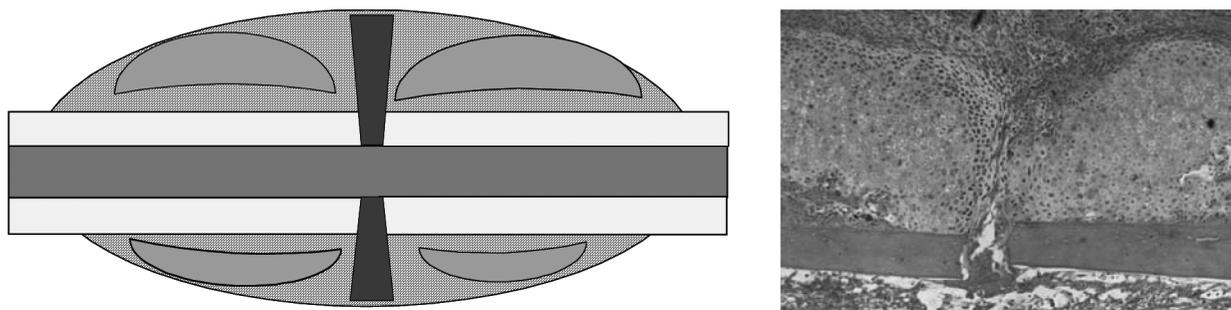


Figure 1. Anatomic characterization of fracture repair. Left panel: diagrammatic presentations of the morphogenetic fields of tissue development and the proximate tissue interactions are presented in sagittal view. Right panel: representative sagittal histological sections of a mid-diaphyseal transverse femur fracture at 14 days. Sections were stained with H&E 100 x magnification.

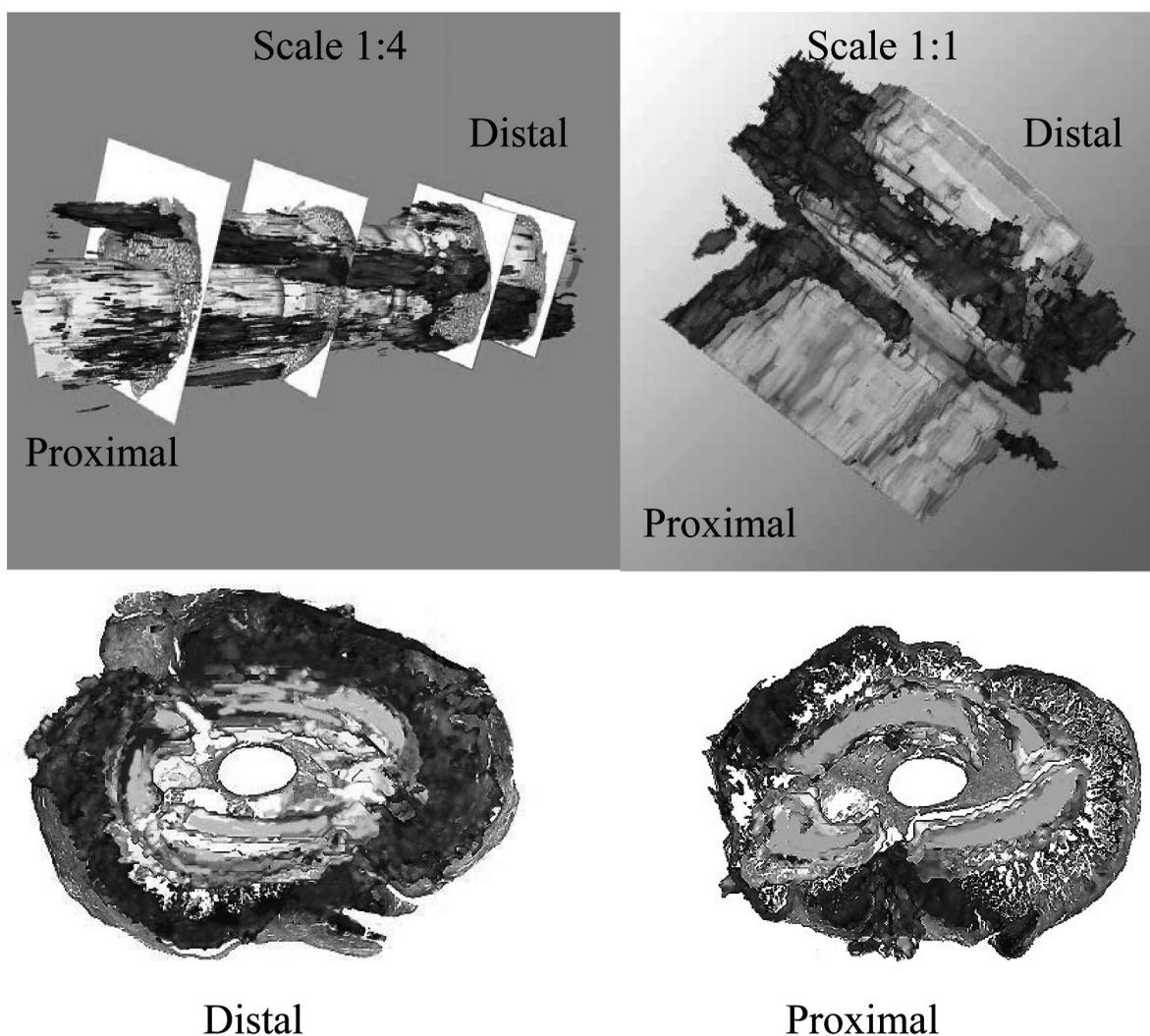


Figure 2. Three-dimensional renderings of mid-diaphyseal simple fractures of a rat femur. Transverse histological sections of rat femur calluses were taken every 100 microns over a distance of 4 mm in both proximal and distal orientations from the fracture line. Sections were reconstructed using Amira software system for 3-D visualization data analysis and 3-D geometric reconstruction (Visual Concepts GmbH, Company, Konrad-Zuse-Zentrum (ZIB), Research Institute, Berlin, Germany). Cartilage tissues are color coded black, cortical bone light gray and the remainder tissue volume gray. Two renderings at an aspect ratio of 1:4 and 1:1 are presented to show the asymmetry of the callus formation. Bottom panels depict single slices at 2 mm from the fracture line in proximal and distal orientations.

STAGES OF FRACTURE REPAIR

Initial Injury Endochondral Formation Primary Bone Formation Secondary Bone Formation
 Inflammation Periosteal Response Cartilage Resorption Coupled Remodeling

RELATIVE TIMES

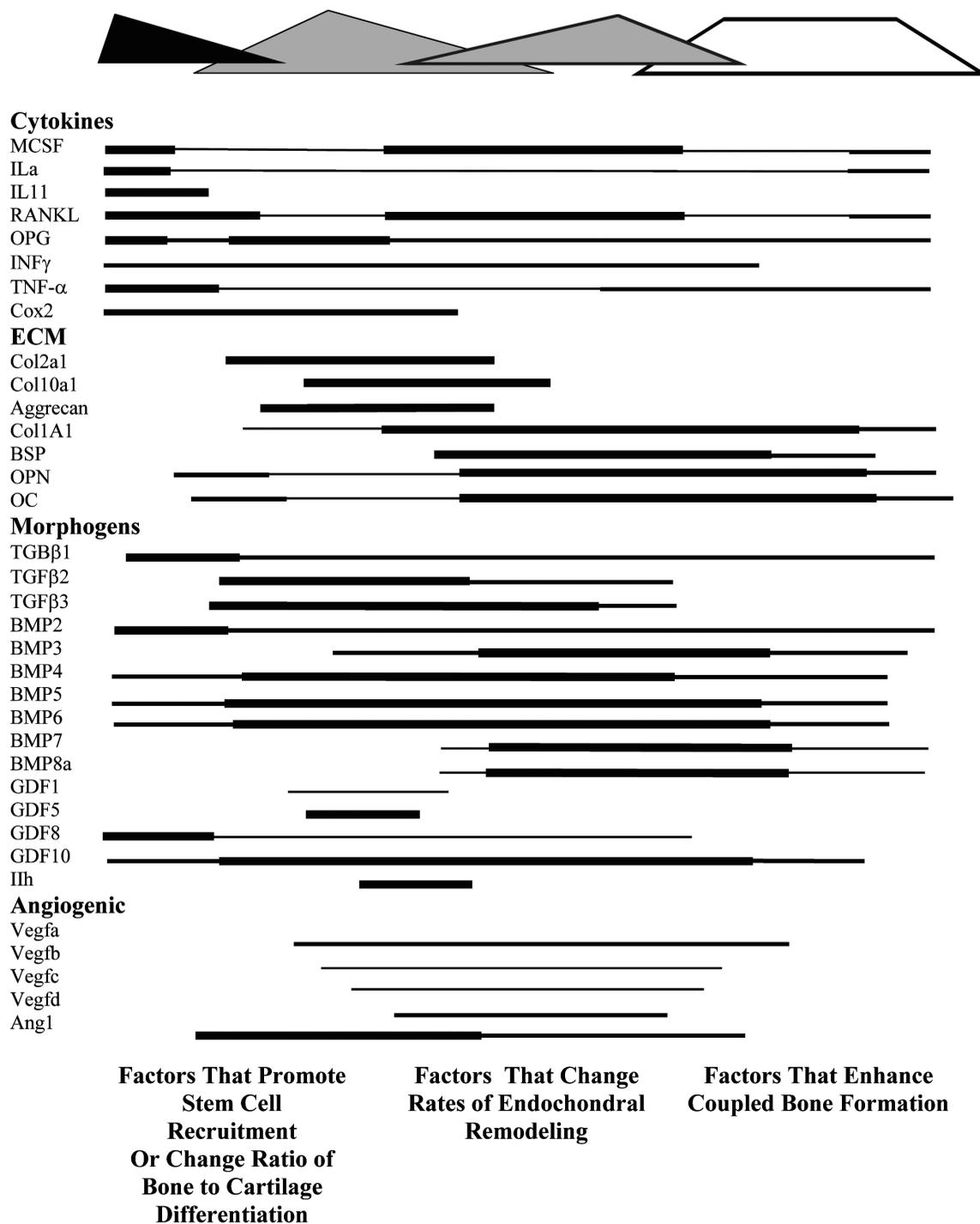


Figure 3. Schematic summary of the stages of fracture repair and their associated molecular processes. The relative temporal aspects of each of the stages of the fracture healing process are denoted by basic geometric shapes that also connote the relative intensity of the molecular processes that define each of the stages. The relative levels of expression of various mRNAs that have been examined in our laboratories are denoted by three line widths. The levels of expression are by percent over baseline for each and are not comparable between individual mRNAs. Data for expression levels for the pro-inflammatory cytokines and the ECM mRNAs;¹⁴ TGF-beta family members²²; proteases and angiogenic factors^{31,32}, Cox2.³⁶ Data pertaining to Ihh is from unpublished data. Time frames and strategies to alter repair at various stages are indicated at the bottom of the figure.

Therapeutic Agent	Effect	Observable Actions	Histology	Other
PGE Like Compound Systemic	Positive	+ BMD, BMC + Strength, + Stiffness / Callus Size	Not Tested	Acutely Toxic
Stem Cells Implantation (Locally injected)	No Effect	+ Callus / Strength, / Stiffness,	Not Tested	Carrier Dependent
BMPs (2 and 7) (Locally injected)	Positive	+ Stiffness + / Strength + / Callus Size	Not Tested	Placement and Carrier Dependent
Coxibs Non-Specific & Specific Systemic	Negative	Stiffness, Strength	+ Cartilage - Bone	Reversible Time Dependent
Statins Local or Systemic	Positive	+BMC, BMD /Strength, Stiffness	Not Tested	Time Dependent
FGFs (Locally injected)	Negative	+ Callus Size - Strength, Stiffness	Not Tested	
PTH Systemic	Positive	/ Callus Size + Strength, Stiffness + BMC, BMD	+ Cartilage + Bone	Persistent Effect Months after Treatment
TGF- β 1 (Locally injected)	Negative	Strength, Stiffness	Not Tested	
Apatite Paste (Locally injected)	Positive	+ Strength, Stiffness	Not Tested	Placement Dependent

* / = no effect, + = positive effect, - = negative effect

Table 1. Effects of various therapeutic agents on fracture healing*.

the cartilaginous callus wraps around the cortical bone towards the medial side as it develops towards the proximal end of callus. Thus, while the signals that initiate the repair either arise from the marrow or are released from the injured bone matrix around the fracture line, the spatial morphogenesis of the callus is regulated by other as yet identified principles that lead to the asymmetry of the tissue development.

One factor that might influence the development of the callus may be the field of inflammatory signals that are propagated from the point of origin of the initial injury⁴. Data supporting the role of inflammatory cytokines in the initiation of skeletal tissue repair are derived from studies showing that in the absence of TNF- α signaling both intramembranous and endochondral bone formation is delayed^{5,6}.

The structural geometry of callus development might also be dependent on the muscular anatomy or vascularization of the tissue as well as the local biomechanical environment at the site of injury. A number of studies have shown that the satellite population of cells in the muscles and muscle precursor cell lines are capable of undergoing skeletal differentiation in response to BMPs or factors released from bone matrix^{7,8} suggesting that the mass of cells that contribute to the callus might be dependent of the surrounding muscular anatomy. Finally it is very clear that the biomechanical environment at the fracture site immensely influences the development of cartilage and bone⁹⁻¹¹.

Questions of how the morphogenetic fields are established and how factors such as the biomechanical environment drive both tissue differentiation and the anatomic geometry of the

regenerative process are of considerable importance in identifying the molecular nature of the initiating signals and relating this to the origins of skeletal stem cells. The answers to these questions also have clinical importance, since the therapeutic responses to bioactive factors may be influenced by the timing and location of their placement into the correct morphogenetic field within the tissue.

Multiple molecular processes contribute to fracture repair

The various stages of fracture repair and the biological processes associated with these stages is presented in Figure 3 and in this same figure the stages at which exogenous factors might be introduced to alter the rate or quality of bone healing are indicated. A summary of the effects of various pharmacological agents that have been examined by our laboratory is presented in Table 1.

a) Pro-Inflammatory Cytokines. As described above, the role of inflammatory cytokines in initiating the repair response is only now becoming fully appreciated, yet the role these molecules play in the regulation of bone remodeling has long been known^{4,12,13}. Two discrete types of resorption take place during fracture repair. The first occurs at the end of the endochondral period in which mineralized cartilage is removed and primary bone formation takes place. M-CSF, RANKL, and OPG are elevated, yet most of the cytokines that have been associated with bone remodeling, including IL1 α , IL β , and IL-6, are absent during this period¹⁴. The exception for this group of cytokines is TNF- α which begins to increase at the end of the period of endochondral resorption. The second type of resorption occurs during secondary bone formation (Figure 3) during which these cytokines become more elevated while OPG, M-CSF, and RANKL show diminished expression levels. These data suggest that the processes that mediate endochondral bone resorption and bone remodeling phases may be different. Foreshortening either endochondral resorption or secondary bone remodeling or enhancing coupled bone will lead to faster repair or earlier regain in bone strength.

b) The Role of the TGF- β Superfamily in Fracture Healing. Since the discovery that demineralized bone induces the *de novo* formation¹⁵ and the subsequent purification of the osteoinductive activity and cloning of the individual bone morphogenetic proteins (BMPs)¹⁶, the TGF- β superfamily of morphogenetic proteins has been perhaps the most intensively studied group of factors in skeletalgenesis and fracture repair^{17,18,19}. Many studies have defined the normal expression of various BMPs during fracture^{20,21}. Recently, our laboratory has shown that specific members of the transforming growth factor- β superfamily, including multiple BMPs¹⁻⁸, GDFs^{1,5,8,10}, and TGF- β 1-3, act in combinations to promote the various stages of intramembranous and endochondral bone formation observed during fracture healing²² suggesting that these factors do not act singly but in a network. While

many current studies have all exogenously introduced BMPs within several days of injury and have all shown that BMPs enhance fracture or bone healing²³, results that have shown that multiple BMPs are produced at high levels for weeks after the initial injury suggest that BMPs might be exogenously administered at multiple stages to promote repair. Future research also is needed to define how combinations of BMPs might interact to optimally enhance healing.

c) Angiogenesis in Fracture Healing. Fracture healing creates a demand on the surrounding tissues to increase blood flow so that induction of bone regeneration can occur within the callus. Such dependency of optimal bone healing on the development of an adequate blood flow has been well established in a number of studies of fracture repair and extensively reviewed²³⁻²⁶. Furthermore, endochondral ossification in normal fracture healing requires the coordination of both the molecular mechanisms that regulate the extracellular matrix remodeling and the vascular penetration of new blood vessels into the resorbing matrix^{27,28}. In a recent study, fracture repair was shown to be enhanced by the exogenous administration of VEGF during the fracture repair process²⁹. These data demonstrate the critical role of VEGF related signaling in neo-angiogenesis and in the endochondral process of new bone formation. The role of the other major class of angiogenic regulators the angiopoietin³⁰ and its contributions in bone repair are not as well understood. Our recent studies show that Ang 1 and the Tie-2- receptor are induced 3- to 5- fold during the initial periods of fracture healing^{31,32}. This indicates that initial vascular ingrowth from feeding vessels in the periosteum may play an important role in the repair process. In this context it is interesting to note that neo-angiogenic vessels that infiltrate the endochondral regions of the callus appear to be fed from the underlying larger vessels that have grown along the cortical surfaces. A number of recent studies have also shown that BMPs will stimulate the expression of VEGF by osteoblasts and osteoblast-like cells^{33,34} and also express VEGF related receptors³⁵. These data demonstrate then that the BMP and VEGF mediated pathways are interrelated and suggest that there is an intimate relationship between them, which would allow for the coordinated regulation of events that initiate new bone formation.

In conclusion, fracture healing offers a unique window into many of the developmental processes that form the skeleton; but in a postnatal context. This may be informative to our further understanding of skeletal growth and repair, as well as those processes which influence skeletal aging.

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