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Cell-Based Therapies for the Treatment of Fractures

Joseph Borrelli, Jr, MD, MBA, Gregory J. Della Rocca, MD, PhD,† Prism Schneider, MD, PhD, FRCSC,‡ Hans-Christoph Pape, MD,§ Paolo Cinelli, MD,§ Yoram Weil, MD,‖ Amal Khoury, MD,‖ Meir Liebergall, MD,‖ and Tracy Watson, MD¶*

Summary: Ongoing studies investigating fracture healing have uncovered and allowed investigators to gain a better understanding of where the variety of cells, which participate in this process, originate, and how they communicate as well as how they can be enhanced to successfully heal a fracture when the process has slowed or failed completely. This brief review will highlight some of the recent findings regarding the role the immune system in fracture healing and how these cells communicate with each other during the healing process. In addition, two 2 methods that have recently been shown to be promising techniques in supporting fracture when it stalls or reversing the process, when the fracture has failed to heal, will also be described.

Key Words: osteoimmunology, bone marrow aspirate, extracellular vesicles, angiogenesis, mesenchymal stem cells

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OSTEOIMMUNOLOGY

There is increasing awareness that the immune system and the skeletal system are intricately linked. The term "osteoimmunology" was coined in 2000 by Arron and Choi[1] to describe this field of study.

Achieving fracture union is dependent on the initial inflammatory phase as the early signaling cascades initiated play a critical role in triggering osseous repair. Both local inflammation and systemic inflammation, mediated by the immune system, play an important role in the activities of osteoblasts and osteoclasts, which affects downstream fracture healing.

The local factors in the initial inflammatory response to fracture include: (1) associated soft-tissue injury, (2) milieu of the fracture hematoma, and (3) early biomechanical stability of the fracture. With extensive local soft-tissue trauma, early fracture healing can be impaired as a result of local blood supply disruption, impaired neutrophil migration to the fracture site, and decreased presence of muscle-derived osteoprogenitor cells.

The early fracture hematomas consist mainly of infiltrated inflammatory cells, which are responsible for regulating local inflammation, and play a very important role in fracture healing (Fig. 1). The acute hematoma is an acidic and hypoxic environment, and this milieu promotes the initiation of a local inflammatory cascade, beginning with the release of proinflammatory cytokines and angiogenic factors.

In addition, the initial vascular and inflammatory response to injury is highly sensitive to mechanical stability and affects osteogenesis. Early stabilization of fractures promotes early vascular response to injury and revascularization throughout the course of fracture repair.[2]

The systemic factors that influence this initial inflammatory response to fracture include the following: (1) acutely elevated systemic inflammation (ie, multiple injuries), (2) chronically elevated systemic inflammation (ie, rheumatoid arthritis and diabetes), and (3) systemic anti-inflammatories (ie, nonsteroidal anti-inflammatories).

Patients with multiple fractures exhibit acutely elevated systemic inflammation, concomitant delayed fracture healing, and increased risk for nonunion.[3] Although neutrophils are vital for early response to injury, neutrophils remain upregulated and primed for up to 2 weeks after major trauma. Prolonged neutrophil presence at the site of fracture hematoma has been implicated in delayed fracture healing.[4]

Chronic inflammatory diseases, such as in diabetes mellitus and rheumatoid arthritis, may cause impaired fracture healing due to factors which increases osteoclastogenesis.[5]

Achieving fracture union is paramount for orthopaedic surgeons; therefore, understanding the interplay between the immune system and the skeletal system is important. It is becoming increasingly apparent that each step along the pathway of providing fracture care can have an impact on fracture union.[6] Strategies surgeons should implement for promoting fracture healing should include the following: realizing the importance of preserving fracture hematoma and the soft tissues about the fracture, providing early fracture stability, minimizing systemic inflammatory response to injury, and optimizing host chronic inflammatory disease states, which often requires a multidisciplinary approach.

CELL-TO-CELL COMMUNICATION AND THE ROLE OF EXTRACELLULAR VESICLES

Although bones possess excellent regenerative properties and most fractures heal uneventfully, approximately

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From the *Department of Orthopedic Surgery and Sports Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL; †Department of Orthopedic Surgery, University of Missouri Hospital and Clinics, Columbia, MO; ‡Departments of Surgery and Community Health Sciences, Cumming School of Medicine, Foothills Medical Centre Calgary, University of Calgary, Calgary, AB, Canada; §Department of Trauma, University of Zurich, Zurich, Switzerland; ‖Orthopaedic Trauma Unit, Hadassah Hebrew University Hospital, Jerusalem, Israel; and ¶Orthopaedic Trauma Service, The CORE Institute, University of Arizona College of Medicine, Phoenix, AZ.

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Reprints: Joseph Borrelli Jr, MD, MBA, 5767 Windber Court, Palm Harbor, FL 34685 (e-mail: Jborthodoc58@gmail.com).

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5%–10% of fractures are complicated by delayed healing or nonunion.[7] Fracture healing is a complex process, regulated by many genes and cell types and influenced by cytokines, chemokines, growth factors, and other molecules. The intercellular communications between cells are important and can occur through either adhesion or direct interactions to transmit signals and modulate physiologic activities or through soluble mediators. Recent studies demonstrated that extracellular vesicles (EVs) also can act as mediators in intercellular communications.[8] EVs are membranous vesicles, which are distinguished from one another by subcellular origin, size, content, and the formation mechanism. Three types of EVs are known: *exosomes*, *shedding microvesicles*, and *apoptotic bodies*.[9] *Exosomes* are cup-shaped membranous EVs, with a diameter of 30–100 nm. *Shedding microvesicles* are generated by blebbing and shedding of the plasma membrane from almost all cell types (dia. 100–1000 nm); *apoptotic bodies* are membrane vesicles generated by apoptotic or dying cells (dia. 50–5000 nm).

EVs carry several kinds of molecular constituents (cargoes) of their cell of origin such as proteins, RNA, DNA, and lipids, and membrane-bound molecules at their surface.[10] EVs also contain proteins related to osteoclast differentiation, such as RANKL and RANK.[11] Furthermore, a number of bone-related microRNAs (miRNAs) have been found to play an important role in osteoblastic differentiation.[12]

The emerging role of EVs in bone remodeling during physiologic and pathologic conditions has been highlighted in a number of recent studies and reviews.[13] It has been shown that during fracture healing, osteoblasts directly communicate with osteoclasts, bone marrow aspiration concentrates (BMACs), and others through EVs. During early phases of fracture healing (coagulation and inflammatory), the activation of platelets, neutrophils, and macrophages play essential roles.[14] A short time after blood vessel and tissue injury, procoagulatory platelet-derived EVs are released, which activate and attract neutrophils and macrophages. The activation of neutrophils, in turn, results in secretion of neutrophil EVs that contribute to increase of the inflammatory response. EVs from BMACs and chondrocytes have been shown to release multiple growth factors, providing a microenvironment conducive for angiogenesis and tissue regeneration.

Various approaches have been developed to enhance fracture healing, including the use of osteogenic materials (bone marrow grafting and injection of active substance), tissue engineering, and stem cell transplantation. These observations open the possibility to use EVs in tissue engineering. The biological characteristics and particular structure of EVs make their use an appealing strategy for tissue regeneration. This novel strategy resolves the problems of immunogenicity and toxicity because BMACs-derived exosomes maintain the immune privileged properties of their cell of origin.

The continuing research on the role of EVs in the fracture-healing process could deliver essential information for improving the treatment of bone fractures. Nevertheless, further studies are necessary before stem cell–released EVs can be developed into a practical and effective therapeutic tool.

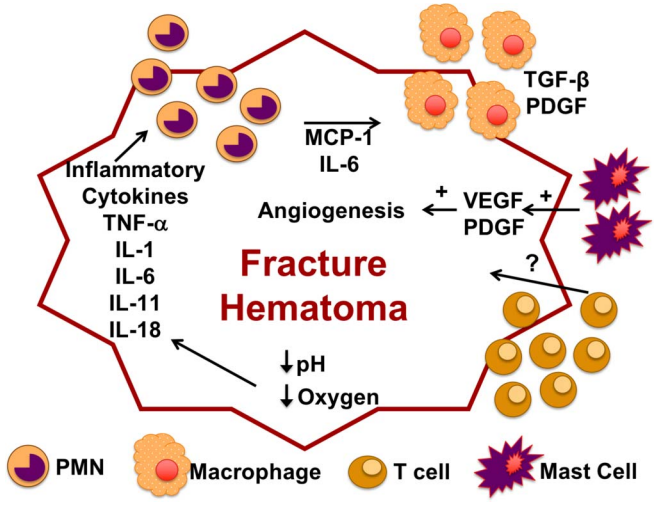

FIGURE 1. The importance of the fracture hematoma in immune mediation of angiogenesis and fracture healing. Inflammatory cytokines include tumor necrosis factor—alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-11 (IL-11), interleukin-18 (IL-18), and monocyte chemoattractant protein-1 (MCP-1). Macrophages produce transforming growth factor—beta (TGF-β) and platelet-derived growth factor (PDGF) and the vascular endothelial growth factor (VEGF) and PDGF released by the mast cells recruited to the fracture hematoma all interact in concert to promote local angiogenesis and downstream osteogenesis.

CELL THERAPY FOR PREVENTION OF DELAYED FRACTURE UNION

Early, painless, and expedite return to function are the goals when treating long bone fractures. In the lower extremity, significant paradigm shift occurred with the development of reamed, locked nailing of femoral and tibial fractures.[15] However, the incidence of delayed and nonunion, especially in tibial diaphyseal and distal metaphyseal fractures, has been reported to be as high as 33%.[16] Recently, biological treatments such as BMAC, and systemic metabolic agents have been investigated as to their ability to facilitate fracture healing.[17]

Risk factors have been identified for impaired fracture healing, and these include fracture morphology, comminution, and the presence of an open fracture. Other host factors such as diabetes, smoking, alcohol consumption, and medical comorbidities have also been associated with the risk of delayed union/nonunion. Newer assessment tools, such the radiographic union scale in tibial (RUST) fractures score and the use of computerized tomography, have significantly increased the ability to more objectively assess fracture healing.[18]

MINIMALLY INVASIVE BIOLOGICAL INTERVENTIONS

When either delayed union or nonunion is diagnosed, several minimally invasive interventional options are

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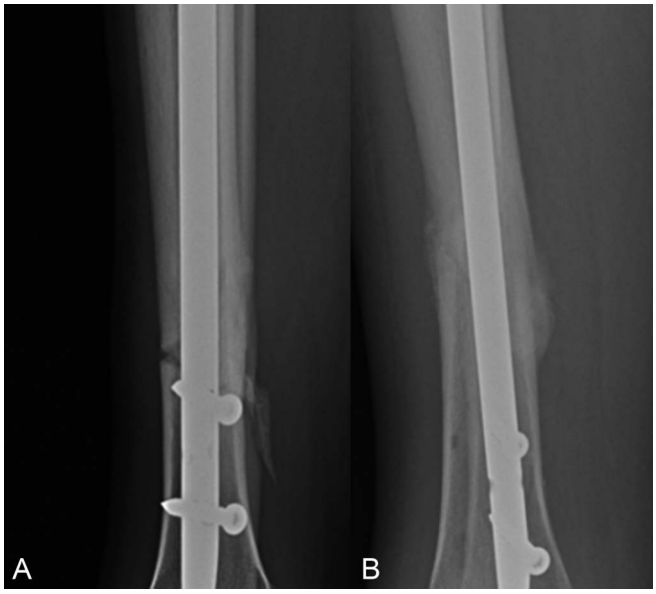

FIGURE 2. Lateral radiographic views of distal tibial fractures fixed with an intramedullary nails. A, Lateral radiograph of a control group patient at 6 months postoperatively, demonstrating still evident fracture gap of the anterior cortex. B, Lateral radiograph of a treatment group patient at 3 months postoperatively showing cortical bridging of both anterior and posterior cortices, abundant callus formation and disappearance of the fracture gap.

available. Bone marrow aspirate injection into a nonunion has had some degree of success in the past.[19] A breakthrough was made with the discovery of the mesenchymal stem cell (MSC) and their osteogenic potential.[20] These MSCs are found in bone marrow as well as other tissues and are identified by the presence of certain surface antigens. BMAC injections have been investigated previously to assess this techniques effectiveness in supporting healing.[17]

Although cells are the essential osteoinductive component of fracture healing, other elements are also involved in the process, including growth factors and matrix proteins. Therefore, a combination of cells, growth factors, scaffold proteins, and a stable mechanical environment ("diamond concept") is believed to be crucial for supporting unimpaired fracture healing.[21]

After reviewing the risks of developing a nonunion and its considerable consequences, we raised the question of whether early, minimally invasive intervention could prevent the development of a nonunion in "at-risk" fractures.

The risk for nonunion of distal diaphyseal tibial fractures has been estimated between 6% and 33%. Therefore, it seemed logical to reduce this risk by early, prophylactic intervention for these fractures. Therefore, we conducted a randomized, prospective study, of an early intervention of distal tibia fractures consisting of injecting MSCs (CD105+) in conjunction with demineralized bone matrix (DBM) and platelet-rich plasma (PRP) into the fracture site in an effort to prevent the development of a nonunion.[22] The treatment group underwent bone marrow and peripheral blood aspiration. Separation of MSCs from the bone marrow aspirate was performed based on the CD105+ antigen. The separation process resulted in the acquisition of a nearly pure MSC subpopulation, these MSCs were mixed with PRP, and the MSC/PRP mixture was then mixed with DBM and injected into the fracture site. At 1 year postoperatively, all fractures healed. However, in the control group, there were 3 cases of delayed union. The most encouraging finding was the significant reduction in mean time to union from 4.0 months in the control group to 2.2 months in the treatment group ($P < 0.03$). Figure 2 represents a typical case comparison between the 2 groups.

Fracture nonunion is a serious consequence of many long bone fractures. Every effort should be made to prevent the occurrence of nonunion. Controllable factors are mainly good surgical technique and providing an adequate biological and mechanical environment for the fracture to heal. However, prompt identification of fractures at risk of developing into a delayed or nonunion will permit minimally invasive procedures to be used that could reverse the pathway of impaired fractured healing.

BONE MARROW ASPIRATE CONCENTRATE FOR FRACTURES AND NONUNIONS

Osteogenic cellular therapies are being developed to include autologous harvesting, cellular concentration, and point of service delivery in the operating room. This technology provides osteogenic material that can be used to stimulate fracture and nonunion healing.

The critical component necessary to promote fracture healing is to provide viable osteoprogenitor cells. Bone marrow is a plentiful source of musculoskeletal stem cells, which can also be found in periosteum, cartilage, muscle, fat, and vascular pericytes.[23] However, there is a paucity of osteoprogenitor cells present in the mature marrow aspirate.[24] Osseous regeneration is dependent on the number of cells available to participate in bone synthesis.

Hernigou et al reported on patients treated for a noninfected nonunion who had undergone bone marrow aspiration from both iliac crests.[25] He demonstrated complete healing in 53 of 60 patients. Those patients who healed had >1500 progenitors/cm^3 while those patients who did heal all had *lower numbers and concentrations of colony-forming units*.

The aspiration technique is very specific to maximize the number of effective progenitor cells per unit volume. Muschler et al[26] determined that no more than 2 mL of blood should be aspirated from any single area in the iliac crest to avoid dilution with peripheral blood. In addition, these transplanted cells must have the appropriate substrate to become attached to once the cells have been implanted at the injury site.

The concept of composite grafts combining marrow elements with other osteoconductive and/or osteoinductive substrates has become a major area of investigation. Loading these cells onto an osteoconductive substrate provides the cells with a stable and subsequently well-vascularized environment. In this environment most of these cells will differentiate into osteoblasts.[27–29]

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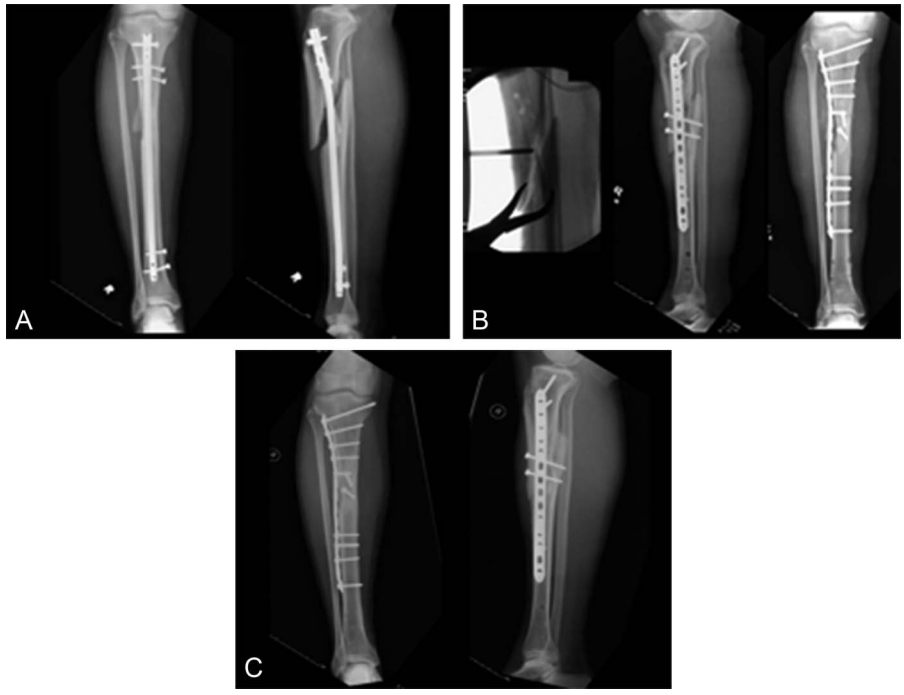

FIGURE 3. A, Anterior posterior (AP) and lateral radiographs, 10 months after intramedullary nailing of a type 3 open tibia fracture, a large fracture gap is present without evidence of fracture healing. B, At revision surgery, the nail was removed with take down of the nonunion site injection of BMAC + DBM directly into the nonunion gap, open reduction, and internal fixation with a plate and screws. C, AP and lateral radiographs demonstrating complete healing of the nonunion at 5 months. Subsequent radiographs at 17 months postoperatively demonstrate excellent remodeling and the absence of the fracture site.

Collagen sponges, hydroxyapatite substrates, and other porous ceramics, as well as particulate DBM have all been used in combination with BMAC to fabricate "composite grafts" (Fig. 3).

Contemporary clinical series have demonstrated excellent results when the above factors have been attended to in the treatment of long bone nonunions. Unfortunately, these combination grafts not enjoyed widespread use. This may be due to (1) the variability resulting from inconsistent and incorrect aspiration techniques and faulty instrumentation necessary to achieve consistent aspirates, (2) low osteoprogenitor content of these insufficient aspirates, and (3) the combination of these aspirates with suboptimal scaffolding materials.

Studies that use correct aspiration and concentration methodologies as well as adhere to using appropriate documented composite grafting techniques do demonstrate the value of marrow aspirates for graft substitution. The efficacy of this technique is related to the number of progenitors cells present in the graft mixture, and highlights the need to concentrate the aspirates and achieve the baseline number of cells necessary to achieve osteogenesis.

CONCLUSIONS

Fracture healing is a highly complex and regimented process. It is now understood that process includes the body's immune system and a complex manner by which the cells participating in fracture healing communicate. Recognizing fractures early on that are not likely to heal or have not healed, allows for interventions, including the use of the body's own osteoprogenitor cells and growth factors with the addition supportive substrates, to stimulate complete healing, thus saving the patient from the considerable morbidity associated with nonunion.

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