

Inflammation, Bone Healing and Osteonecrosis: From Bedside to Bench

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Abstract: Osteonecrosis of the epiphyseal and metaphyseal regions of major weight-bearing bones of the extremities is a condition that is associated with local death of bone cells and marrow in the afflicted compartment. Chronic inflammation is a prominent feature of osteonecrosis. If the persistent inflammation is not resolved, this process will result in progressive collapse and subsequent degenerative arthritis. In the pre-collapse stage of osteonecrosis, attempt at joint preservation rather than joint replacement in this younger population with osteonecrosis is a major clinical objective. In this regard, core decompression, with/without local injection of bone marrow aspirate concentrate (BMAC), is an accepted and evidence-based method to help arrest the progression and improve the outcome of early-stage osteonecrosis. However, some patients do not respond favorably to this treatment. Thus, it is prudent to consider strategies to mitigate chronic inflammation concurrent with addressing the deficiencies in osteogenesis and vasculogenesis in order to save the affected joint. Interestingly, the processes of inflammation, osteonecrosis, and bone healing are highly inter-related. Therefore, modulating the biological processes and crosstalk among cells of the innate immune system, the mesenchymal stem cell-osteoblast lineage and others are important to providing the local microenvironment for resolution of inflammation and subsequent repair. This review summarizes the clinical and biologic principles associated with osteonecrosis and provides potential cutting-end strategies for modulating chronic inflammation and facilitating osteogenesis and vasculogenesis using local interventions. Although these studies are still in the preclinical stages, it is hoped that safe, efficacious, and cost-effective interventions will be developed to save the host's natural joint.

Keywords: chronic inflammation, osteonecrosis, osteogenesis, vasculogenesis, bone healing, inflammation

Introduction

Inflammation: General Principles

Acute inflammation is the first step of the healing of all tissues and organs subjected to physical (mechanical), chemical, infectious, thermal, and other types of injurious stimuli. Such trauma leads to activation of the innate immune system, and subsequent release of cytokines, chemokines, reactive oxygen species, and other pro-inflammatory stimuli, and triggering of the complement and coagulation systems.^{1,2} These events are initiated by the recognition of specific chemical motifs called pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) on/within the cells at the site of injury.³ PAMPs are derivatives of infectious organisms. DAMPs are molecular byproducts from dead or dying cells and are also referred to as endogenous

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danger signals. The most important PRRs include the Toll-like Receptors (TLRs), the C-type lectin receptors, the NOD-like receptors, the RIG-I-like receptors, and others.³ The acute inflammatory response of the innate immune system is a generalized broad response to eradicate or negate the offending stimulus, initiate the clearing of cellular debris, and begin the resolution and reconstruction of normal host tissue. Interestingly, the phase of repair and renewal is aided by the pro-inflammatory environment, that in the case of musculoskeletal and many other tissues, activates or licenses mesenchymal stem cells (MSCs) and endothelial progenitors to migrate to the injured area under the direction of chemokine gradients.^{4–8} Acute inflammation can lead to restoration of native host tissue, fibrosis, or chronic inflammation.

Chronic inflammation is a persistent injurious state in which acute inflammation and fibrosis continue, despite ongoing unsuccessful attempts at definitive resolution and repair.^{1,9,10} In simple terms, innate immune processes (and if applicable the more restricted antigen-specific adaptive immune system) cannot overcome the offending adverse stimulus to reconstitute normal anatomy and physiology.^{11,12} Thus, homeostasis is never achieved despite the continued mobilization of all biological resources. Chronic inflammation is also a state of heightened energy demands, in which organelles such as the mitochondria, endoplasmic reticulum, and other important components of the cell become exhausted, inefficient, dysfunctional, and dysregulated.^{13–15} If chronic inflammation persists, the resilience and survival of the organism are at risk.

The cellular profile of the innate immune system comprises cells of the monocyte/macrophage lineage, especially local DAMP and PAMP sensing macrophages, polymorphonuclear leukocytes (neutrophils), dendritic cells, mast cells, specific lymphocyte subgroups including NK cells, and other cell types. Chronic inflammation involves the above cells as well as other T and B cell subgroups. Fibroblasts and vascular lineage cells appear in both acute and chronic inflammatory states. During the resolution of inflammation, pro-inflammatory M1 macrophages polarize to an anti-inflammatory, pro-reconstructive, pro-vascularization M2 phenotype, with reciprocal effects of local mesenchymal and vascular progenitors.^{1,16,17}

Osteonecrosis: Definition and Etiology

Osteonecrosis encompasses a diverse set of conditions that lead to the death of the bone cells and marrow within

a bone compartment.^{18–20} Osteonecrosis can be localized or widespread (multifocal). For simplicity, osteonecrosis of the femoral head (ONFH) will be used as the prototype condition. Many different predisposing factors are associated with osteonecrosis. In general, ONFH is due to traumatic events (eg, a displaced fracture of the femoral neck, hip dislocation, or vigorous attempts at closed reduction of a dislocated hip) or may be atraumatic, ie, not due to mechanical injury. Traumatic etiologies are thought to compromise the vascular supply to the local area directly. Atraumatic etiologies include the use of high dose corticosteroids, excessive alcohol intake, autoimmune diseases such as systemic lupus erythematosus (SLE), radiation, chemotherapy, hypercoagulable states, sickle cell disease, Gaucher's disease, and other causes.^{21,22} Osteonecrosis usually afflicts the epiphyseal and metaphyseal areas of bone, and can lead to the collapse of bone and secondary degenerative arthritis. Osteonecrosis must be differentiated from insufficiency fractures due to overuse, pathological fractures in abnormal bone, and other conditions. Osteonecrosis often occurs in the large weight-bearing joints of the hip (femoral head), knee (femoral and tibial condyles), and humerus (head), but can occur in virtually any bone and location. The majority of cases are associated with corticosteroid use or alcohol abuse, usually in younger patients in their prime working years.^{23,24} Collapse of the involved joint advances to painful and debilitating end-stage arthritis. Therefore, it is important to diagnose osteonecrosis early so that potential inciting factors can be assessed and mitigated, limiting progression to the later stages. Furthermore, early diagnosis and treatment may possibly arrest or reverse the progression of disease, thereby retaining the patient's own anatomical structures and avoiding joint replacement surgery. Unfortunately, a recent study reported from our tertiary care center with a special interest in osteonecrosis disclosed that 77% of cases were diagnosed in the late stages of ONFH, compromising joint saving procedures.²⁵

Relationship Between Chronic Inflammation and Osteonecrosis

Despite the fact that numerous etiologies are associated with osteonecrosis, the final pathway involves inadequate oxygen and nutrient supply to the affected area (Figure 1). These events are associated with enhanced differentiation of MSCs along the adipogenic pathway, and deficient osteogenic and vasculogenic pathways.^{22,26} The lesions

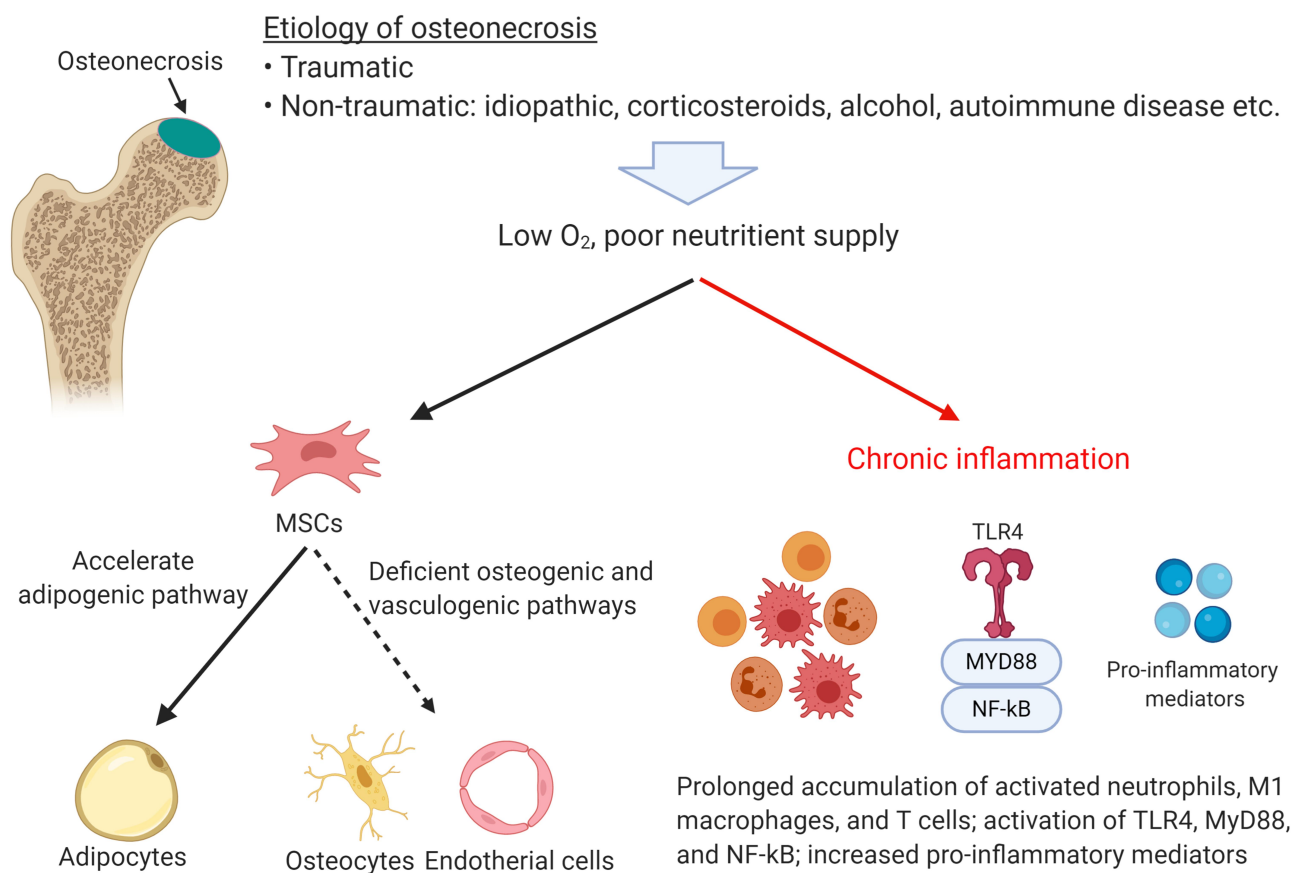


Figure 1 Pathophysiology of osteonecrosis. Numerous etiologies are associated with osteonecrosis. However, the final common pathophysiologic pathway involves inadequate oxygen and nutrient supply to the affected area. These events lead to enhanced differentiation of MSCs along the adipogenic pathway, and deficient osteogenesis and vasculogenesis. Osteonecrosis also demonstrates signs of chronic inflammation: persistent accumulation of activated neutrophils, macrophages, T cells, and other cell types; continued activation of TLR4, MyD88, and NF-κB; increased production of pro-inflammatory mediators.

Abbreviations: MSCs, mesenchymal stem cells; TLR, toll-like receptors; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B.

of multifocal osteonecrosis are often diagnosed at various stages of the disease. However, at some point, the affected anatomical areas demonstrate histological evidence of chronic inflammation, cell death, and compromised resolution and repair.²⁷ Real-time image probe analysis shows that 6 weeks after induction of osteonecrosis by vascular cauterization in mice, activated macrophages and neutrophils persist locally.²⁷ In other studies, steroid-associated osteonecrosis in rats resulted in upregulation of the PRR Toll-like Receptor 4 (TLR4), the downstream adapter protein for the majority of TLRs: Myeloid differentiation factor 88 (MyD88), the major transcription factor for inflammatory proteins: Nuclear Factor-Kappa B (NF-κB), and Monocyte Chemoattractant Protein-1 (MCP-1).²⁸

It is appreciated that many of the molecules related to acute and chronic inflammation, osteonecrosis, and bone healing are overlapping, and play major roles in activation of the innate immune system and tissue repair. NF-κB is the major pro-inflammatory transcription factor induced by

injurious stimuli; pro-inflammatory factors activate or license MSCs.¹ TLR4 is a PRR on the cell surface, and is activated by PAMPs, DAMPs, and other substances.²⁹ TLR4 has two signaling pathways: the MyD88 dependent (TLR4/MyD88/NF-κB) pathway and the MyD88 independent (TLR4/TRIF/IRF3) pathway.³⁰ The MyD88 dependent pathway activates NF-κB and promotes the expression of MCP-1, a chemokine.³¹ MCP-1 is a chemoattractant for cells of the monocyte-macrophage lineage and the MSC-osteoblast lineage.³² MCP-1 induces the proliferation of monocytes/macrophages and promotes the differentiation and activation of osteoclasts.³³ In a porcine model, byproducts of necrotic bone have been shown to upregulate numerous pro-inflammatory cytokines in a mechanism that is dependent on TLR4 activation by macrophages.³⁴ This observation has been confirmed in a rat model of steroid-associated ONFH, which demonstrated excessive activation of TLR4/NF-κB and suppression of the canonical Wnt/β-catenin pathway

(the latter pathway regulates cell fate, cell migration, and organogenesis).³⁵ In a study in which serum was collected from 20 patients with various stages of ONFH and compared with serum from normal controls, eight genes, including TLR4 were identified as potential serum biomarkers of the disease.³⁶ The other biomarkers including BIRC3, CBL, CCR5, LYN, PAK1, PTEN, and RAF1 were related to inflammation, bone and cartilage metabolism, and vasculogenesis. This suggests that potential biological strategies to mitigate the adverse sequelae of osteonecrosis might entail curtailing chronic inflammation, and facilitating bone formation and vasculogenesis.

Strategies to Mitigate Chronic Inflammation and Enhance Osteogenesis and Vasculogenesis in ONFH

Healing of chronic critical-size bone defects due to trauma (delayed union, nonunion), previous infection, periprosthetic osteolysis, and other causes is similar, in many ways, to defect that are encountered in osteonecrosis. To a lesser or greater degree, all of these etiologies have a component of chronic inflammation with localized bone necrosis, fibrosis, deficient osteogenesis and vasculogenesis, and fatty infiltration of the tissues. Consequently, research from in vitro and

in vivo models of healing of critical-size bone defects is relevant to treating osteonecrotic lesions. Strategies and methodologies that have been used to solve these difficult clinical scenarios from our laboratory and others will be reviewed in this light.

Inhibition of Chronic Inflammation

Given the fact that osteonecrosis is associated with chronic inflammation, it seems prudent to consider interfering with these processes. Potential approaches must be delivered in a temporal and spatially sensitive manner, as soft and hard tissue healing after acute injury is dependent on a short period (usually several days) of acute inflammation for subsequent resolution and initiation of repair by licensing MSCs and other cells.^{4,5,37-42}

Given these facts, below are possible methods to mitigate chronic inflammation (Figure 2):

- interfering with or obstructing receptor ligation and continued activation that prolongs the inflammatory process,
- inhibiting the relevant pro-inflammatory pathways within the cell,
- impeding the transcription, translation, or release of inflammatory mediators,
- interfering with the end-organ response to specific inflammatory mediators,

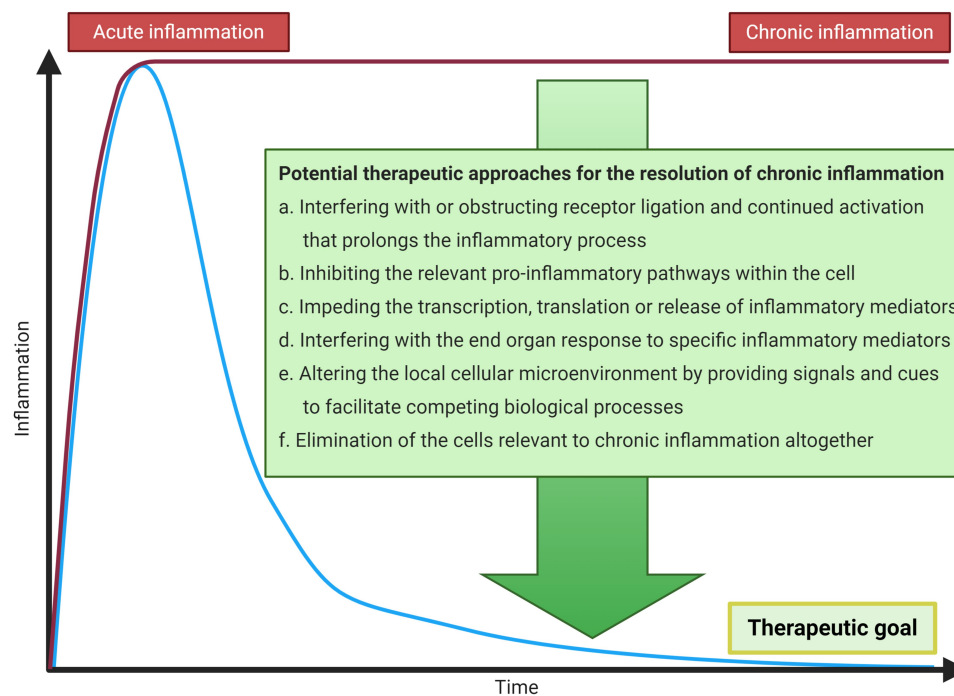


Figure 2 Potential therapeutic approaches for the resolution of chronic inflammation. Acute inflammation is necessary for healing of tissues after injury. However, chronic inflammation is detrimental, and leads to loss of tissue integrity and function. Potential avenues for mitigation of chronic inflammation are listed.

(e). altering the local cellular microenvironment by providing signals and cues to facilitate competing biological processes,

(f). elimination of the cells relevant to chronic inflammation altogether.

Many of these strategies have been used in the treatment of systemic chronic inflammatory diseases such as rheumatoid arthritis (RA). Pharmacologic agents for RA include antimetabolites and other chemotherapeutic agents, disease-modifying drugs and biologics that directly or indirectly interfere with specific cytokines, chemokines, and other pro-inflammatory molecules such as Tumor Necrosis Factor alpha (TNF α), Interleukins (IL) such as IL-1 β and IL-6, etc. Although these drugs are highly efficacious in treating RA, systemic delivery of these medications with potential serious adverse effects is not pragmatic for chronic inflammation due to osteonecrosis and critical-size bone defects.⁴³ Thus, local delivery is probably the preferred route. With respect to mitigating chronic inflammation in clinical scenarios relevant to osteonecrosis and critical-size bone defects, the following local approaches have promise: inhibition of specific TLRs, most prominently TLR4; interference with the following: the adapter protein MyD88, the transcription factor NF- κ B, or the chemokines MCP-1 and Macrophage Inhibitory Factor (MIF); altering the macrophage

polarization state from an M1 pro-inflammatory to an M2 anti-inflammatory phenotype via local delivery of IL-4 or IL-13. Our laboratory and others have utilized some of these strategies in models simulating chronic inflammation associated with wear particle disease.^{41,44–52} Infusion of IL-4, an anti-inflammatory cytokine is one important putative strategy to curtail chronic inflammation in a wide variety of clinical conditions.^{41,53–56} IL-4 protein can be delivered directly, via scaffolds or other devices, or as genetically modified MSCs that over-express IL-4 constitutively, or in response to upregulation of NF- κ B.^{41,48,57–62} This approach is one of the great interests of our laboratory's treatment of chronic bone defects and osteonecrosis.^{1,63} Other potential immunotherapeutic approaches include delivery of IL-13, IL-10, IL-1Ra, TNFsR, etc.⁶⁴

Local Delivery of Biomolecules and/or Cells for Osteogenesis and Vasculogenesis (Figure 3)

Biomolecules and Drugs

Local delivery of growth factors and other molecules that enhance osteogenesis and vasculogenesis, or inhibit osteoclasts for the treatment of bone defects and osteonecrosis is not a new concept.⁶⁵ These factors include members of

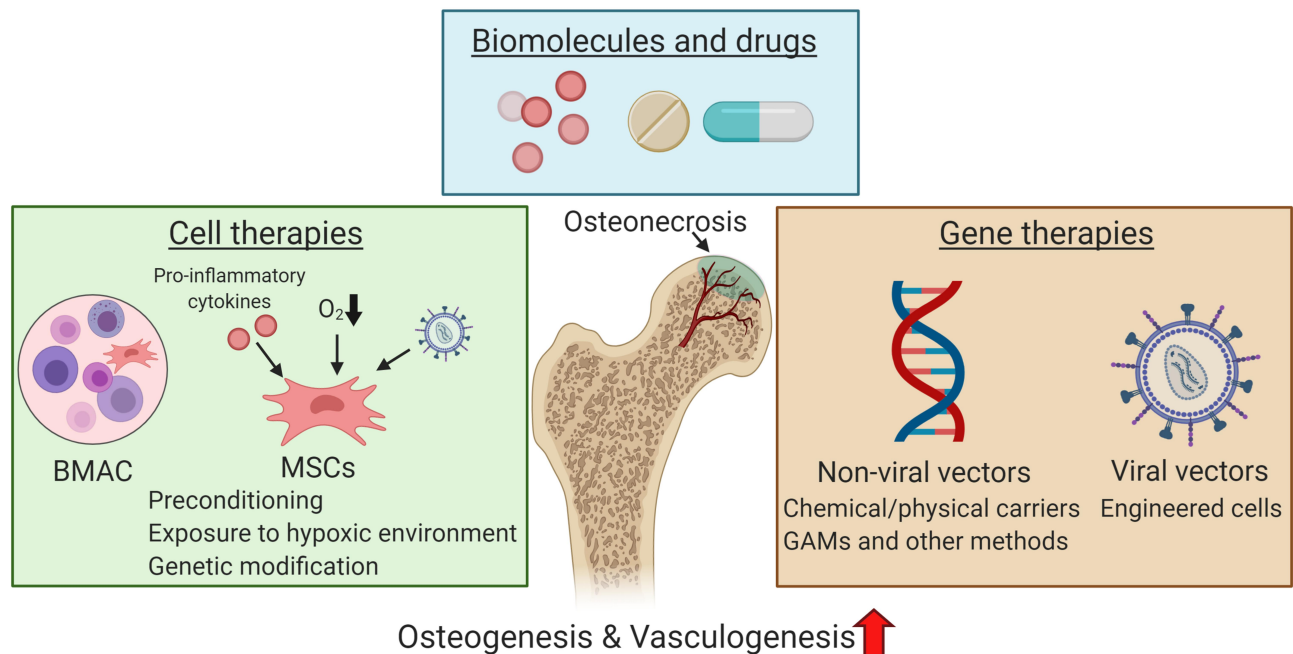


Figure 3 Potential approaches for local delivery of biomolecules, cell therapies, and gene therapies to enhance osteogenesis and vasculogenesis in osteonecrosis. **Abbreviations:** BMAC, bone marrow aspirate concentrate; MSCs, mesenchymal stem cells; GAMs, gene-activated matrices.

the Transforming Growth Factor (TGF) superfamily including TGF β and Bone Morphogenic Proteins (BMPs), Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Insulin-like Growth Factor (IGF), Hepatocyte Growth Factor (HGF), Parathyroid Hormone (PTH), and others.^{66,67} These agents have been delivered locally as proteins within various polymers, scaffolds, types of cements, etc. The biomolecules are absorbed, entrapped, immobilized, or coated as drug delivery systems and then released by diffusion, matrix or crosslinker degradation.⁶⁷ Other drugs for local delivery include corticosteroids and other sterols, statins, and bisphosphonates.^{67,68} These biomolecules are often multi-functional, modulate numerous pathways including the inflammatory cascade, osteogenesis, and vasculogenesis, and have other biological targets. Although some of these interventions have been explored in extensive pre-clinical and limited clinical studies for the healing of critical sized defects, few have been used clinically for the treatment of osteonecrosis.^{69–72} In fact, the clinical trials have not led to widespread acceptance and implementation. Systemic treatment with bisphosphonates or statins has not proven to be effective for ONFH in a recent systematic review, however local treatment may prove to be efficacious.⁷³ The challenges of the necrotic, avascular harsh biological environment in osteonecrotic lesions may be too demanding for pharmacologic therapy alone to be successful.

Cell Therapies

Cell therapy for ONFH and other bones afflicted with this disease is no longer experimental. The use of concentrated autologous iliac crest bone graft in conjunction with core decompression (CD) in the early stages of osteonecrosis is evidenced based.^{18,74–78} Perhaps the most compelling study is the one reported by Hernigou et al in 2018, in which the authors performed simultaneous bilateral CD in 125 sequential patients; in one of the two hips undergoing CD, they added bone marrow aspirate concentrate (BMAC).⁷⁶ The number of MSCs (or colony-forming unit-fibroblasts [CFU-Fs]) injected into the CD site ranged from 45,000 to 180,000 cells with a mean of 90,000 \pm 25,000 cells. After 20–30 years of follow-up, the addition of BMAC was found to reduce the rate of collapse of the femoral head from 72% to 28%; the percentage of patients undergoing hip replacement was reduced from 76% to 24%. Using quantitative MRI, the volume in the femoral

head occupied by the osteonecrotic lesion in the group receiving BMAC decreased from 44.8% to 12%. These are compelling data. The concept of addition BMAC to CD for treatment of ONFH would benefit from a large prospective randomized multicenter study.

Extensive preclinical and laboratory analysis has been performed on BMAC and is reviewed in recent publications.^{74,79–82} Numerous factors are relevant to the number and vitality of the harvested cells, including the age and gender of the patient, the presence of medical comorbidities and medications such as corticosteroids and others, smoking, obesity, etc. It is important to note that BMAC is not MSCs, but a conglomerate of different mononuclear cell types including macrophages, lymphocytes, mast cells, and other cells. In fact, only about 1 CFU-F was present in 30,000 nucleated cells harvested from the anterior iliac crest, which equates to about 600 CFU-Fs per cc of bone marrow aspirate.⁸²

Despite the apparent success of BMAC for the treatment of osteonecrosis, several questions remain. Is BMAC the preferred cells to inject for osteonecrosis? How many cells of different lineages are necessary? Are MSCs alone sufficient? Can MSCs be altered to mitigate inflammation and facilitate healing of the osteonecrotic lesion? Are allograft-derived MSCs equally efficacious? Although no definitive answers are currently available for these questions, some pertinent observations need mentioning. There is substantial evidence that the addition of macrophages will augment the osteogenic capabilities of MSCs, probably by licensing the latter cells and engaging in continuous MSC-macrophage crosstalk to enhance tissue repair.^{17,38,41,53,62,83–85} These findings substantiate injecting an agglomerate of MSCs and hematopoietic lineage cells. Nevertheless, autologous and allogenic MSCs alone (and/or their byproducts such as exosomes) are being isolated, expanded and delivered to heal bone defects and for treatment of osteonecrosis.^{86–90} In the USA, the FDA has rather stringent regulatory guidelines for the use of cells and their byproducts, which must undergo “minimal manipulation” prior to use in humans.⁹¹ A recent publication from the present authors summarizes significant modifications to the phenotype of MSCs (more than minimal manipulation) to facilitate bone healing.⁹ Some of these techniques include preconditioning with biologics, exposure of MSCs to low oxygen environments, and gene therapy/genetic manipulation of cells. Some other approaches include optimizing techniques for isolation, expansion, and storage of MSCs, and improving the

physical, chemical, topographic, electrical, and other properties of the carrier or scaffold used, and the defect in the host into which the cells are implanted.

Gene Therapies

Gene therapy and the genetic manipulation of cells for the purpose of musculoskeletal tissue healing are an exciting concept and have been reviewed elsewhere.^{9,92} Gene therapy may be accomplished using chemical and physical methodologies without viruses to transport DNA or micro-particles into cells; by the use of gene activated matrices (GAMs) or other platforms that support the release of genetic material to the surrounding cells according to pre-determined temporal and spatial parameters; via the use of viral vectors to engineer autologous or allogeneic cells *ex vivo* with subsequent injection of these cells *in vivo*; or by direct transfer of genes into cells *in vivo*.⁹ Gene therapy has also been used as a treatment for osteonecrosis, mostly in preclinical studies.^{93–100} We have used BMAC, MSCs, preconditioned MSCs, and genetically modified MSCs that overexpress IL-4 injected into the CD tract with/without a novel 3D printed, customized functionally graded scaffold as a treatment for ONFH in rabbits.^{1,101} In preclinical studies, the addition of IL-4 over-expressing MSCs in the acute phase of osteonecrosis may hamper regenerative efforts by suppressing the acute inflammatory reaction that is necessary for bone healing. Other strategies are currently being assessed.

Summary

The use of biomolecules, drugs, cells, and gene therapy for the treatment of osteonecrosis is very enticing. However, these treatments are generally in the preclinical stage except for BMAC therapy, and must be weighed against numerous potential risks including unintended adverse effects on neighboring cells, and the development of immunogenicity, mutagenicity, and carcinogenesis. Furthermore, the timing, dose and optimal platform for delivery, and issues related to cost-effectiveness must be addressed.

Discussion

Chronic inflammation is detrimental to all tissues and organs. This process generally leads to the replacement of normal host tissue by an undesirable fibrovascular stromal scar laden with acute and chronic inflammatory cells. This substitute tissue does not have the anatomical, physiological, metabolic, and functional integrity of the host

tissue. In clinical cases in which substantial portions of an organ are afflicted by chronic inflammation, the operational performance of vital processes may be jeopardized, eg, in chronic hepatitis, nephritis, diabetes, cardiopulmonary disease, RA, aging, and other disorders.^{102,103} The associated morbidity and mortality are substantial.¹⁰⁴

In this regard, bones and joints are no different. Chronic inflammation is often seen in inflammatory arthritis, chronic osteomyelitis, nonunion of fractures, and osteonecrosis. This is manifested as persistent unresolved overactivity of the innate, and in some cases, the adaptive immune systems. In osteonecrosis, despite various associated predisposing factors, chronic inflammation in response to DAMPs impedes neovascularization and osteogenesis. This situation will progress to joint collapse and end-stage arthritis if it is not arrested. The situation is even more dire in cases of multifocal osteonecrosis.^{105,106}

The optimal treatment for early-stage osteonecrosis involves strategies for mitigation of chronic inflammation, and fostering of osteogenesis and vasculogenesis prior to joint collapse. In these cases, joint preservation is a much better option than joint replacement, due to the patient's young age. However, the exact treatment for these complex cases, often in the presence of persisting predisposing factors (eg, continued high dose corticosteroids for the treatment of SLE) sometimes restricts the medical practitioner's options.

Systemic pharmacological approaches appear to have little utility in the treatment and prevention of osteonecrosis in the adult.⁷³ Early diagnosis is important so that treatment options can be reviewed and implemented. This suggests that high-risk patients need to be identified and screened, at least with a comprehensive history and possibly with selective non-invasive imaging such as MRI.²⁵

Local treatment with CD, possibly with the addition of biological adjuncts such as BMAC seems reasonable in the pre-collapse stages. Research should address what component(s) of the BMAC and what doses optimize reconstitution of the osteonecrotic defect. Specific biological approaches might focus on the issues of attendant chronic inflammation, and their adverse effects on osteogenesis and vasculogenesis. Custom design of cells and biologics derived from the patient's own tissues, though currently not approved by the FDA as they would involve more than minimal manipulation, might further improve the aims and goals of regenerative medicine for osteonecrosis. Custom-designed mechanically based implants

could potentially delay physical collapse of bone and cartilage, and provide important signaling cues for tissue regeneration. Such implants that are 3D printed and biodegradable are currently being tested in preclinical studies in our laboratory.¹⁰¹ It is hoped that some of these technologies will prove to be safe, efficacious, and cost-effective. In this way the pain, disability, and morbidity of the millions of patients with osteonecrosis worldwide might be assuaged.

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Disclosure

Prof. Dr. Stuart B Goodman reports a patent for a customized load-bearing and bioactive functionally graded implant for the treatment of osteonecrosis issued to Stanford University. The authors report no other conflicts of interest in this work.

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