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The role of the adaptive immune system in burn-induced heterotopic ossification and mesenchymal cell osteogenic differentiation

*Kavitha Ranganathan, MD, Shailesh Agarwal, MD,[1] David Cholok, BS,[1] Shawn Loder, BS, Jonathan Li, MD, Hsiao Hsin Sung Hsieh, DDS, Stewart C. Wang, MD, PhD, Steven R. Buchman, MD, and Benjamin Levi, MD**

Department of Surgery, University of Michigan Health Systems, Ann Arbor, Michigan

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ABSTRACT

Background: Heterotopic ossification (HO) is the pathologic process of extraskeletal bone formation. Although the exact etiology remains unknown, inflammation appears to catalyze disease progression. The goal of this study is to determine the impact of the adaptive immune system on HO.

Methods: HO was induced in 8-wk-old control C57BL/6 and immunocompromised Rag1tm1Mom (Rag1 KO) male mice deficient in B- and T-lymphocytes via combined Achilles tenotomy and burn injury. Microcomputed tomography quantified the extent of HO formation at the tenotomy site. Adipose-derived mesenchymal stem cells were harvested to evaluate osteogenic differentiation potential.

Results: Areas of developing HO demonstrated substantial enrichment of CD45 + leukocytes at 3 wk after injury. HO from Rag1 KO mice was substantially less mature with foci of cartilage and disorganized trabecular bone present 12 wk after injury. Rag1 KO mice formed 60% less bone compared to immunocompetent controls (4.67 ± 1.5 mm *versus* 7.76 ± 0.65 mm; $P = 0.001$). Tartrate-resistant acid phosphatase staining and immunofluorescent analysis of osteoprotegerin and nuclear factor kappa-light-chain-enhancer of activated B cells demonstrated no appreciable difference in osteoclast number or activation. Alizarin red staining *in vitro* demonstrated a significant decrease in osteogenic potential in immunocompromised mice compared to controls (29.1 ± 0.54 mm *versus* 12.1 ± 0.14 mm; $P < 0.001$).

Conclusions: We demonstrate a prominent role for the adaptive immune system in the development of HO. In the absence of mature B- and T-lymphocytes, HO growth and development are attenuated. Furthermore, we demonstrate that mesenchymal populations from B- and T-cell deficient mice are inherently less osteogenic. This study identifies a potential therapeutic role for modulation of the adaptive immune system in the treatment of HO.

* *Corresponding author*. Department of Surgery, University of Michigan, 1500 E Medical Center Drive, Ann Arbor 48105, MI. Tel.: +1 937 684 7192; fax: +1 734 936 4000.
E-mail address: blevi@med.umich.edu (B. Levi).

[1] These authors contributed equally.

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Introduction

Heterotopic ossification (HO) is the pathologic formation of ectopic bone within extraskeletal structures commonly occurring as a result of musculoskeletal trauma, severe burns, neurologic injury, and orthopedic intervention.[1-5] With improved critical care, the incidence of HO is rising with improved survival after major trauma.[2-5] Sequelae of post-traumatic HO, including chronic pain, poor wound healing, and joint contracture, represent a significant barrier to functional recovery and independence. Treatment, including surgical excision, is limited, and most patients never regain complete range of motion due to persistent or recurrent contractures.[3,6]

Osteoimmunology, or the study of the immune system in bony metabolism, is an area of growing interest in the study of HO. The incidence and severity of HO after trauma strongly with the degree of injury and resultant inflammation.[1-4,7,8] Several inflammatory and/or infectious stimuli have been demonstrated to positively contribute to ectopic bone formation.[8-12] Data from fracture healing suggest a central role for the immunologic cytokine cascade in remodeling and repair after injury.[13] Contributions from the innate or nonspecific immune system to HO have previously and repeatedly been demonstrated in trauma models.[14-16] Furthermore, in genetic models, such as fibrodysplasia ossificans progressiva, histologic analysis of early lesions demonstrates the presence of a lymphocytic infiltrate suggesting a potential immunologic origin to even atraumatic ectopic bone pathologies.[17]

While it is likely that the immune system plays a role in HO, the exact nature of this relationship is unclear. Early inflammatory markers interleukin (IL)-3, IL-12, and IL-13 have been associated with HO development after trauma.[18] The presence of HO also correlates with higher levels of IL-6, IL-10, monocyte chemoattractant protein-1, and IL-10.[18] Furthermore, the complement cascade has been implicated in the process of HO.[19] These cytokines likely regulate both osteoblastogenesis and osteoclastogenesis suggesting a complex interplay between immunologic signals and bone formation.

Given the persistent nature of ectopic bone and its associated cytokine signals, the adaptive immune system is a potential target around which to study immunologic contributions to HO. Both B- and T-lymphocytes have demonstrated effects on bone formation in the regulation of mesenchymal stem cell (MSC) differentiation via interferon-γ and tumor necrosis factor-α, and inhibition of osteoclastogenesis through release of osteoprotegerin (OPG).[20-23] In this study, we sought to identify whether the adaptive immune system and more specifically B- and T-lymphocytes, contribute to the formation and progression of ectopic bone after trauma.

Methods and materials

Animals

Eight-wk-old male mice were used for all studies. Mice carrying a Rag1 knockout mutation (C57BL/Rag^{T1Mom}; $n = 4$) preventing the maturation of both B- and T-lymphocytes were matched to C57BL/6 immunocompetent controls ($n = 4$). Microcomputed tomography (MicroCT) and histologic analysis were performed at 12 wk after induction of HO. A separate group of C57BL/6 ($n = 3$) mice wasused to visualize inflammation present at earlier time points coinciding with future sites of HO formation.

All animal procedures were carried out in accordance with the guidelines provided in the *Guide for the Use and Care of Laboratory Animals* from the Institute for Laboratory Animal Research (ILAR, 2011) and were approved by the Institutional Animal Care and Use Committee of the University of Michigan (PRO0001553).

Burn and tenotomy

All mice received a partial thickness burn injury. Briefly, animals were anesthetized with 3%-5% inhaled isoflurane. Hair was clipped on the left dorsum to expose the skin. A partial thickness burn was by placing a metal brand heated to 60°C in a water bath against the exposed skin for 18 s. Each mouse received a concurrent Achilles tenotomy with aseptic, sharp dissection at the midpoint in the left hind limb. Pain management was achieved with subcutaneous injections of buprenorphine every 12 h for 3 d.

μCT analysis

In vivo development of bone formation was assessed with longitudinal μCT scans at 12-wk after injury (μCT: Siemens Inveon, using 80kVp, 80 mA and 1100 ms exposure). Images were reconstructed, and ectopic bone volume was calculated using total Hounsfield units to visualize the full extent of ectopic bone formation. All scans were also analyzed with threshold Hounsfield units (HU) of 800 (total), 1250 (intermediate density), or 1800 (high density) to determine the volume of tissues. Samples were then further analyzed to look at the arrangement of ectopic tissue in relation to the site of tenotomy. Abnormal bone adjacent to the calcaneus was defined as distal HO while ectopic tissue present in muscle or soft-tissue areas and not immediately adjacent to native bone was defined as proximal HO. Analysis of the scans was performed by a blinded investigator to minimize bias.

Histologic processing and analyses

At 1-, 3-, and 12-wk postoperatively animals were euthanized for histology.[24] Skin was removed from the tenotomy site and the hind limb was then fixed in buffered formalin solution for 24 h at 4°C. Decalcification of the sample was completed with 19% ethylenediaminetetracetic acid solution for 28-42 d at 4°C. Decalcified tissues were dehydrated through graded ethanol and paraffin embedded. Transverse sections were completed with a width of 5 μ, mounted on Superfrost plus slides (Fisher Scientific, Pittsburg, PA), and dried overnight at 37°C. Histology including H&E, Safranin-O, and aniline blue stains were performed to visualize the anatomy and maturity of bone at the site. Tartrate-resistant acid phosphatase staining was performed to determine the role of osteoclasts in the evolution of the deposited HO.