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The Immunological Contribution to Heterotopic Ossification Disorders

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Abstract

The formation of bone outside the endogenous skeleton is a significant clinical event, rendering affected individuals with immobility and a diminished quality of life. This bone, termed heterotopic ossification (HO), can appear in patients following invasive surgeries and traumatic injuries, as well as progressively manifest in several congenital disorders. A unifying feature of both genetic and non-genetic episodes of HO is immune system involvement at the early stages of disease. Activation of the immune system sets the stage for the downstream anabolic events that eventually result in ectopic bone formation, rendering the immune system a particularly appealing site of early therapeutic intervention for optimal management of disease. In this review we will discuss the immunological contributions to HO disorders, with specific focus on contributing cell types, signaling pathways, relevant in vivo animal models, and potential therapeutic targets.

Keywords

Heterotopic Ossification; Immune System; Complement; Macrophages; Mast Cells; Lymphocytes; Cytokines; Chemokines; Fibrodysplasia Ossificans Progressiva; Progressive Osseous Heteroplasia

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Conflict of Interest

MR Convente, H Wang, RJ Pignolo, FS Kaplan, and EM Shore all declare no conflicts of interest.

Human and Animal Rights and Informed Consent

All studies by the authors involving animal and/or human subjects were performed after approval by the appropriate institutional review boards. When required, written informed consent was obtained from all participants.

Introduction

Heterotopic ossification (HO), or bone formation at extraskeletal sites, is a serious medical condition that can occur as a result of trauma [1–3] or as a consequence of genetic mutations as occur in Fibrodysplasia Ossificans Progressiva (FOP), Progressive Osseous Heteroplasia (POH), and Albright's Hereditary Osteodystrophy (AHO) [4, 5].

Common, non-genetic forms of HO, such as those that occur as a result of high impact blast injuries or hip arthroplasty, may develop in patients with no clear genetic predisposition [1–3, 6]. Epidemiology studies have documented HO development following trauma as a significant complication in 12% to 25% of fractures [7], and recent reports from the military suggest that as many as 60% of traumatic blast injuries have associated HO [8]. A major contributing factor to both genetic and non-genetic forms of HO is the immune system, as implicated by the clinical observation that inflammatory events trigger, and in some cases, predict HO development [9–16], suggesting that the etiology of HO formation is uniform across multiple types [3, 14, 15, 17].

FOP and POH are two severe genetic forms of HO. In both, the extra-skeletal bone formation does not occur during embryonic development, but typically begins during early childhood. Patients with FOP or POH typically develop their first HO episode during the first decade of life [5, 18]. As each disease progresses, patients develop additional HO lesions that can severely restrict mobility and greatly impact quality of life. In POH, which is caused by inactivating mutations of the *GNAS* gene [18], heterotopic ossification first forms in more superficial tissues such as the dermis, occurs through an intramembranous ossification pathway, and has not been correlated with trauma or an inflammatory response. Heterotopic bone formation in FOP, caused by gain-of-function mutations in the BMP receptor Activin A Receptor, Type 1 (ACVR1; also known as ALK2) [19], shows similarities to non-hereditary trauma-induced HO, with HO primarily forming within soft connective tissues through endochondral ossification and association with a robust inflammatory response at the initial stages of the bone induction process [17].

The reciprocal interactions between bone and the immune system have gained substantial interest in recent years, and an inflammatory stimulus appears critical in at least some forms of heterotopic ossification. Why certain disorders of extraskeletal bone formation involve inflammatory triggers while others do not is a key question remaining in the field. Nevertheless, for those HO disorders that do involve an inflammatory component, it is vital for researchers and physicians to better understand how inflammation is involved in order to more effectively treat or prevent these diseases.

In this article, we will review the contributions of the immune system to the formation of HO, and discuss the relevance of the early inflammatory component as a possible therapeutic target for treatment of HO.

Bone Biology and the Immune System

Effects of the immune system on bone biology, termed “osteimmunology” [20], have been reported during embryological and post-natal skeletal development, fracture repair, bone

homeostasis, and heterotopic ossification [13, 15, 17, 21–26]. Notably, excessive inflammation impairs embryological [27] and post-natal [26] skeletal growth, and directly leads to cytokine-mediated bone resorption [28]. By contrast, robust heterotopic (extra-skeletal) ossification is often initiated within a strong inflammatory microenvironment [11–14]. Long bone fracture repair involves massive inflammation to repair the endogenous skeleton and requires both anabolic and catabolic functions of the immune system during the healing process [21, 24]. Perhaps most striking is that the pro-inflammatory factors implicated in skeletal bone loss during chronic inflammation [28] are also identified as predictive of HO development [12, 13, 15], indicating dual consequences of the immune system in bone biology.

The Immune System

The mammalian immune system, which evolved as a defense against pathogens and endogenous injury, is composed of two branches – the innate immune system and the adaptive immune system. The innate immune system, which acts as a first responder to infection and injury, utilizes several families of particle recognition receptors such as toll-like receptors (TLRs) and nucleotide oligomerization domain-like (NOD) receptors to provide a short-term barrier against pathogens and injury [29]. The adaptive immune system provides long-term protection against pathogens, utilizing gene rearrangement mechanisms to produce antigen-specific responses to foreign invaders [30]. Each branch performs largely independent functions, though both branches communicate using intermediary cells such as natural killer (NK) cells. Broader functions of the immune system that extend beyond pathogen defense involve diverse processes such as muscle repair, wound healing, fibrosis, fracture repair [21, 24, 31–33], and HO development [3, 10, 13–15, 34**, 35*]. Both branches of the immune system have been implicated in HO formation; key findings of immunological contribution to HO formation and bone biology can be found in Table 1.

Innate Immunity and Heterotopic Ossification

Recent reports implicate at least three components of the innate immune system in HO formation - the complement system, macrophages, and mast cells.

Complement System and HO

The complement system is a signaling pathway of the innate immune system that aids in recognition of pathogens by myeloid-derived cells, such as neutrophils and macrophages [36]. The primary roles of complement include opsonization of pathogens, chemotaxis of neutrophils and macrophages via upregulation of the cytokine/chemokine network, disruption of pathogen cell membranes, and interaction with coagulation pathways. More recently, the complement system has been appreciated for its involvement with bone regeneration and repair [23]. In a genetic association study between single nucleotide polymorphisms (SNPs) and HO, the less common SNP variant of toll-like receptor 4 (TLR-4) and complement factor H were associated with a decreased risk of HO [7]. Complement factors C3a and C5a modulate osteoclast formation and the inflammatory response of osteoblasts synergistically with IL-1 β , and complement C3 and C5 deficiency impairs callus formation and new bone growth in a model of fracture repair [22, 37]. These

studies, which link an early inflammatory response to fracture repair and pathological HO formation, highlight the importance of an inflammatory contribution in the development of heterotopic bone.

Macrophages

Macrophage precursors have the ability to form osteoclasts in response to certain cytokines, such as receptor activator of nuclear factor kappa-B ligand (RANKL) [38]. However, a subset of bone-lining macrophages, termed OsteoMACs, has also been shown to exhibit pro-osteogenic function in tibial fracture repair models, highlighting the capacity of macrophages to aid in bone formation under normal repair processes [21, 24]. Additional in vitro studies demonstrated that macrophages can produce BMP2 to activate osteo-inductive signals [39]. In these studies, human mesenchymal stem cells grown in conditioned media from J774A.1 macrophage cells increased expression of specific osteogenic genes such as alkaline phosphatase. This induction was blocked by antibodies against either BMP2 or transforming growth factor-beta1 (TGF- β 1) [39].

Macrophage contribution to HO has also been noted in vivo. Macrophages are capable of secreting numerous pro-inflammatory cytokines and chemokines [40], including factors associated with blast-injury-induced HO development, such as IL-6, IL-10, and monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) [13]. Robust macrophage accumulation has been observed in episodes of cardiac HO [41], and both FOP patients and animal models of the condition exhibit extensive infiltration of macrophages at early stages of HO lesion formation [42*, 43].

The strongest evidence for macrophage contribution to HO formation comes from studies in which macrophage populations are depleted. Kan et al [44] utilized a transgenic mouse model of HO in which BMP4 is over-expressed in cells with an actively-expressed *Nse* promoter [45]. Depletion of macrophages reduced ectopic bone formation by up to 75% [44], suggesting that injury-recruited macrophages mediate the injury response by secreting osteogenic factors, including BMP4, which in turn trigger HO formation [42*, 44]. While it is clear that macrophages have a critical role in HO development, whether specific macrophage populations, such as M1 and M2 macrophages [46], contribute differently to the development of HO remains undetermined.

Mast Cells

Mast cells are hematopoietically-derived tissue resident cells distributed throughout the vascularized tissues and serosal cavity. Mast cells were originally recognized for their roles in allergy response and anaphylaxis [47]. However, over the last several years many new functions of mast cells with much broader roles in biology have been identified, including tissue edema, wound repair, scar formation, fibrogenesis, angiogenesis, pain pathophysiology, and tumor invasion [48–59].

Recently, with the *Nse-BMP4/MOR* (–/–) double mutant mice, in which μ -opioid receptor (MOR)-null mice over-express BMP4 in cells with an active *Nse* promoter, Kan et al [35*] showed resulting attenuated mast cell activation and documented a significant reduction in

HO formation in response to injury. Additional studies further showed that opioid signaling may play a key role in mast cell activation and the downstream inflammatory responses associated with HO. Moreover, mast cells can be recruited and activated by the potent neuro-inflammatory molecules Substance P (SP) and calcitonin gene related peptide (CGRP), whose levels are elevated following BMP2 induction [60]. SP and CGRP, which are involved in the integration of neuropathic pain, were also shown to be required for HO development in several animal models. Inhibition of the SP and CGRP signaling pathways, accomplished via numerous genetic and pharmacological methods, abolished HO formation in animal models of HO development [10, 34**, 61].

The first evidence that mast cells may be involved in the pathology of FOP came from biopsy analysis of lesion tissue acquired from patients. Gannon et al [62] showed that mast cells are present at every developmental stage of FOP lesions and are most pronounced at the highly vascular fibroproliferative stage. Mast cell density at the periphery of FOP lesion tissue is 40- to 150-fold greater than in normal control skeletal muscle or in uninvolved skeletal muscle from FOP patients and 10- to 40-fold greater than in any other inflammatory myopathy examined [62]. Subsequently, the presence of mast cells in a mouse model of FOP (Acvr1 R206H) [42*] was demonstrated. Substance P, shown to be integral for HO formation in animal models, is also highly expressed in early stage lesion tissue from FOP patients [34**].

Mast cell involvement has also been documented in non-genetic HO disorders, strongly suggesting the cell type is critical in the overall pathology of HO. Analysis of cardiac and peritoneal HO revealed presence of mast cells near sites of ectopic bone formation [41, 63]. Mast cells have also been shown to directly induce fibrosis following biomaterial scaffold implants in mice, indicating a more universal role of these cells in the overall wound repair process [56, 64, 65].

These comprehensive data strongly suggest a paramount role for mast cells in the overall pathology of HO and indicate mast cells as a major therapeutic target. As with macrophages, the most compelling evidence that mast cells are critical for HO development comes from the aforementioned mast cell ablation animal models.

Adaptive Immunity and Heterotopic Ossification

Cells of the adaptive immune system – T cells and their subsets, B cells, and antigen-presenting cells [66] – evolved primarily as a defense against pathogen infection. Complex signaling pathways, such as the interleukin family [67] and interferon family [68] of cytokines, act in tandem to protect against acute and chronic infectious disease. This branch of the immune system also serves as an integral component in early HO development. Shared cell types and signaling pathways across multiple forms of HO indicate a unified and significant role for adaptive immunity in the pathology of ectopic bone development.

Lymphocytes

Previous studies have reported an abundance of lymphocytes in early-stage lesions in FOP, both in patients [14, 69] and animal models [42*]. Lymphocyte populations are among the

earliest infiltrating cells in FOP lesions [17], suggesting a role for establishing a microenvironment conducive to downstream catabolic events. The accumulation of T and B cells observed in FOP lesions is associated with destruction of skeletal muscle that precedes ectopic cartilage and bone formation [69, 70]. In instances of non-genetic HO development, cells of lymphoid origin have also been identified in regions adjacent to ectopic bone. Histopathological investigations of bone formation in cardiac valves documented the presence of T and B cells in close proximity to ectopic bone [41, 71]. No evidence for the involvement of antigen-presenting natural killer (NK) cells in HO formation has been reported to date.

Cytokines/Chemokines and Heterotopic Ossification

Cells of the immune system communicate via complex signaling pathways that govern the pro-inflammatory microenvironment of involved tissues. Strikingly, a subset of pro-inflammatory cytokines and chemokines repeatedly appears to be upregulated in instances of non-genetic HO. Serum levels of specific interleukin family cytokines IL-6, and IL-10 [13] and effluent levels of IL-6 and IL-13 [15] were significantly increased in blast injury patients with HO compared to patients that did not develop ectopic bone. Patients who developed HO following total hip arthroplasty also exhibited elevated levels of IL-6 in addition to several other interleukin family cytokines [12]. Interferon gamma (IFN- γ) was also elevated in these patients. In vitro studies involving T cell and human mesenchymal stromal cell (hMSC) co-culture revealed a subset of cytokines (TNF- α , TGF- β , IFN- γ , and IL-17) capable of inducing endogenous BMP2 expression in hMSCs and promoting hMSC differentiation and mineralization [72]. Serum levels of MCP-1, a chemokine known to attract T cells [73] and monocytes [74], was also significantly higher in blast injury patients with HO [13] and detectable in patients who developed HO following total hip arthroplasty [12].

Chemokine and cytokine signaling plays a significant role in recruitment of fibroproliferative cells during wound healing and fibrosis, and this interaction could be a major foundation for immunological involvement in HO formation. Mesenchymal stem cells express a repertoire of functional chemokine receptors and are actively recruited following injury as a cell source in wound healing responses [75–77]. Interestingly, both mesenchymal stem cells and fibroblasts are capable of producing their own chemokines, as well as responding to autocrine and exogenous chemokine signals [32, 78]. Direct contribution of several cytokines has been documented in fibrotic disease [31, 79, 80] and is consistent with a scenario in which episodes of HO, particularly those caused by blast injury and FOP, can be reasonably described as aberrant wound healing. For non-genetic HO disorders, the contribution of chemokines and cytokines extends to fibroproliferative cell recruitment. In FOP, contributing fibroproliferative cells exhibit cell-autonomous enhanced chondrogenic potential [81*], and the wound healing process is likely to be further corrupted.

The subset of cytokines and chemokines identified above exhibit a wide range of immunological functions, as all except IL-10 function as pro-inflammatory mediators that potentiate cell activation, differentiation, maintenance, and chemotaxis [74, 82]. It is important to highlight the role that BMP signaling plays in further potentiating the pro-

inflammatory state of lymphocytes, as both genetic and non-genetic incidents of HO can manifest as a result of overactive BMP signaling. BMP4 ligand affects early T cell development by acting on both T cell precursors and the thymic stroma [83]. The BMP signaling pathway is also active in peripheral lymphocytes [84], which suggests a broader immune-regulatory role of BMP signaling in these cells. T cells express several BMP Type I and Type II receptors, including ACVR1 [85], the receptor mutated in FOP [19]. In this context, T cells are capable of cell autonomous up-regulation of BMP signaling, which may potentiate the local pro-inflammatory microenvironment absent of any exogenous ligands. Acting more globally, administration of recombinant human-BMP2 in the treatment of unicameral bone cysts produced an exaggerated inflammatory response in patients, suggesting a universal ability of BMPs to induce inflammation [86]. Additionally, BMP2, in tandem with osteogenin, acts as a strong chemoattractant for monocytes and induces their expression of TGF- β 1, leading to further recruitment of progenitor cells involved in endochondral ossification [87].

Despite strong evidence of immunological involvement in HO development, no evidence exists for mature immune cells or their hematopoietic precursors participating as chondro/osteoprogenitor cells to the fibroproliferative, chondrogenic, or osteogenic stages of HO [70]. Furthermore, ablation of mature T and B cells, accomplished in a double-transgenic *Nse-BMP4; Rag1^{-/-}* mouse, did not prevent injury-induced HO formation, though the rate of HO formation was diminished [70]. These data emphasize that the immunological contribution to HO development consists of an overall enhanced inflammatory microenvironment and recruitment of separate chondro/osteoprogenitor cells, as opposed to hematopoietically-derived cells directly forming ectopic cartilage and bone. Thus, targeting the immune system has strong therapeutic potential to mitigate lesion progression in HO disorders and potentially reduce associated symptoms and disability.

Therapeutic Intervention in HO Disorders - Targeting the Immune System

There is a pressing need to develop therapeutics for genetic and non-hereditary HO disorders, as current treatment options are predominantly palliative [6, 17, 88]. Innovative approaches targeting the chondro- and osteogenic stages of HO progression have so far proven successful in mouse models of heterotopic endochondral ossification [11, 89**]; however, these treatments may not be allowable for extended use in humans due to potential deleterious effects on the endogenous skeleton and other tissues. Targeting the immune system for therapeutic intervention offers several advantages – treatment directed at the earliest stage of HO development, and availability of a large set of FDA-approved drugs. Below, we will review treatment options currently in use, as well as discuss possible new methods for targeting the immune system.

Current treatment options targeting the immune system in HO disorders incorporate the use of broad immunosuppressive drugs. For patients undergoing hip arthroplasty, preoperative radiation of the hip region and postoperative treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) are established methods to prevent heterotopic ossification [90]. The mechanism of action of NSAIDs is based on cyclooxygenase (COX) inhibition, which subsequently decreases synthesis of prostaglandins that are key for bone formation [91]. In

line with the previous report, selective COX-2 inhibition prevents heterotopic ossification after hip replacement [92]. Remarkably, a single FOP patient treated with global immunosuppressive agents for graft-versus-host disease following a bone marrow transplant did not develop any additional ectopic bone throughout the entire 14-year duration of treatment; HO returned after treatment was discontinued [70]. These reports suggest that targeting the immune system can be successful at inhibiting HO formation; however, drugs that target specific immune cells or pathways may be favorable to existing options.

Ablation of specific immune cell populations is likely to be reserved for proof-of-principle experiments demonstrating the requirement of certain immune cells for HO development [10, 34**, 44], however it is worth reviewing one pharmacological method by which this is accomplished. Van Rooijen [93] describes a protocol to apoptotically ablate mature macrophages via liposome-encapsulated clodronate, taking advantage of the phagocytic activity of the cell. This ablation technique has been utilized numerous times in animal models successfully [93–96]. However, due to the remaining pool of hematopoietic stem cells, macrophages will repopulate within a few days to weeks after cessation of clodronate liposome delivery, depending on delivery method [93], and long-term administration of clodronate liposomes has not been conducted. Nevertheless, the technique has been attempted in humans and was well tolerated and successful [97], suggesting that temporary ablation of macrophages may be a pursuable option for treating HO disorders.

The involvement of mast cells and the Substance P (SP) pathway in HO pathology is well supported. SP acts on mast cells via binding to the NK-1R receptor, which induces mast cell degranulation and release of numerous pro-inflammatory factors [98]. Pharmacological inhibition of mast cells and SP is possible in two ways. The NK-1R receptor can be directly targeted by the NK-1R antagonist aprepitant, which has been successfully used to inhibit intracellular calcium ion flux, a readout of SP signaling [99]. Inhibition of mast cell degranulation can be accomplished by administration of cromoglicic acid (also known as cromolyn), an FDA-approved drug for asthma indications. Cromolyn treatment was successful at significantly reducing bone formation in a BMP2-implant mouse model of HO [10], and although its existing FDA approval status may make this a viable option for patients, its current routes for administration result in poor systemic distribution. An additional option involves using the c-kit tyrosine kinase inhibitor imatinib to induce mast cell apoptosis, which has proven successful at reducing inflammation associated with rheumatoid arthritis [100] and decreases HO in an Achilles tendon injury model of HO [101*].

The use of biologics to target specific cytokines and chemokines is a very appealing treatment option for HO disorders. Biologics are commonly monoclonal antibodies or receptor decoys that act to sequester excessive signaling ligands, resulting in reduced molecular signaling levels while providing significant therapeutic benefit [102]. These reagents have a proven record of success in treating a wide range of inflammatory disorders, including rheumatoid arthritis [103, 104], and are widely available, comprising over 30% of licensed drugs [102]. Biologics that target TNF- α , interleukin family members, and other pro-inflammatory factors implicated in HO pathology may provide significant clinical benefit to patients predisposed to ectopic bone formation. Additionally, since biologics are

extremely specific, side-effects commonly experienced during global immunosuppression therapy may be avoided.

Genetic disorders of HO offer additional means for therapeutic intervention, as patients with FOP, POH, or AHO possess specific mutations that can be targeted. Interventions by such approaches are less likely to be restricted to the immune system, though the signaling pathways altered in these genetic disorders are active in immune cells. Small molecule inhibition of ACVR1/ALK2, the Type I BMP receptor mutated in FOP, has been successful in an FOP mouse model [11]. A downside to this approach is that currently available BMP signaling inhibitors tend to act non-specifically, targeting multiple BMP receptors. Fortunately, optimized versions of BMP signaling inhibitors that have specific affinity for ACVR1/ALK2 are in development [105] and may eventually provide clinical benefit with minimal off-target effects.

Conclusions

The link between the immune system and HO development has been shrouded in mystery. Only during the last several years has research started to unravel the details of this connection. Although immunological contributions to HO have been previously observed, these observations were mostly reserved to characterizing immune cell populations present in early HO lesions. As the importance of inflammation to HO development has become more apparent, detailed investigations of the cells and signaling pathways responsible for disease pathology are starting to uncover the critical inflammatory factors that directly contribute to HO formation.

The current body of literature speaks to a stimulatory effect of the immune system on adjacent and recruited chondro/osteoprogenitors in the development of HO, as opposed to direct cellular contributions to ectopic cartilage and bone. Considering the major role that inflammation plays in wound healing and tissue repair, and that many episodes of HO are induced following injury, one could reasonably describe HO as arising from an abnormal tissue repair and wound healing program. This may result from an overall elevated pro-inflammatory microenvironment, enhanced recruitment of wound healing fibroblasts, a shift in the normal tissue repair and wound healing mechanisms toward ectopic cartilage and bone formation, or a combination of these and other abnormalities. Ongoing experiments are elucidating these possibilities.

Perhaps the most promising aspect of the above studies is the wealth of potential therapeutic targets for the treatment of HO that have been illuminated. Immuno-ablation proof-of-principle experiments have begun to identify the immune cell populations critical for HO formation, and additional studies are identifying specific inflammatory molecules predictive and causative for ectopic bone development across multiple HO disorders. Treating HO at its earliest inflammatory stage offers the possibility that lesion progression will be minimized and perhaps halted before maturation into chondro-osseous tissue.

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Table 1

Key findings of immunological contribution to heterotopic ossification and bone biology

<u>Innate Immunity</u>	Complement	-SNP variant (CC vs. TT) in complement factor H associated with HO -Deficiencies in complement factors C3 and C5 resulted in decreased callus area and reduced new bone formation in fracture repair	[Mitchell et al., 7] [Ehrnthal et al., 22]
	Macrophages	-Depletion of macrophages by clodronate treatment reduced HO volume by 75% -Macrophages express osteo-inductive and HO-associated signaling factors -Bone-lining OsteoMAC subset population promotes in vivo bone healing	[Evans et al., 13] [Alexander et al., 21] [Kan et al., 44] [Champagne et al., 39] [Mosser et al., 40]
	Mast cells	-Robust mast cell density in early lesion tissue of FOP, cardiac HO, and peritoneal HO patients -Ablation/blockage of mast cells significantly reduced HO volume	[Salisbury et al., 10] [Kan et al., 34**] [Mohler et al., 41] [Gannon et al., 62] [Di Paolo et al., 63]
<u>Adaptive Immunity</u>	Lymphocytes	-Earliest responding cells in early lesions in FOP and cardiac HO patients	[Shore and Kaplan, 17] [Mohler et al., 41]
<u>Cytokines/Chemokines</u>		-HO-associated factors identified in blast injury and hip arthroplasty patients -Chondro/osteogenic progenitor cells that participate HO development are recruited to sites of injury via chemokine signaling	[Hoff et al., 12] [Evans et al., 13] [Forsberg et al., 15] [Ishida et al., 32] [Smith et al., 78]