Review

Roles of High Mobility Group Box 1 in Cardiovascular Calcification

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Key Words

High mobility group box 1 • Cardiovascular calcification • Calcific extracellular vesicles • Osteochondrogenic differentiation

Abstract

Calcific disease of the cardiovascular system, including atherosclerotic calcification, medial calcification in diabetes and calcific aortic valve disease, is an important risk factor for many adverse cardiovascular events such as ischemic cardiac events and subsequent mortality. Although cardiovascular calcification has long been considered to be a passive degenerative occurrence, it is now recognized as an active and highly regulated process that involves osteochondrogenic differentiation, apoptosis and extracellular vesicle release. Nonetheless, despite numerous studies on the pathogenesis of cardiovascular calcification, the underlying mechanisms remain poorly understood. High mobility group box 1 (HMGB1), a nuclear protein bound to chromatin in almost all eukaryotic cells, acts as a damage-associated molecular pattern (DAMP) when released into the extracellular space upon cell activation, injury or death. Moreover, HMGB1 also functions as a bone-active cytokine participating in bone remodeling and ectopic calcification pathogenesis. However, studies on the roles of HMGB1 in promoting cardiovascular calcification are limited to date, and the mechanisms involved are still unclear. In this review, we summarize recent studies investigating the mechanism of cardiovascular calcification and discuss multiple roles of HMGB1 in its development.

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Introduction

The entire cardiovascular system is vulnerable to pathological calcification [1], and vascular disease, such as atherosclerosis, arteriosclerosis and diabetic vasculopathy, is frequently complicated by calcification [2]. Calcification in arteries can be widespread, and the prevalence increases with age. Indeed, more than 60% of adults over the age 60

years have calcium deposits in at least one of the major vascular beds, such as the carotid arteries, coronary arteries and intrathoracic aorta [3]. Cardiovascular calcification leads to a multitude of clinical manifestations. Intimal calcification, which is always associated with atherosclerotic plaque, can lead to myocardial infarction or ischemia in both coronary and peripheral arteries. In larger arteries such as the aorta, medial calcification can lead to increased pulse wave velocity and systolic hypertension, a known risk factor for cardiovascular disease in the general population. Another common site of calcification is the aortic valve, termed calcific aortic valvular disease (CAVD), which has severe consequences. Approximately 40% of people over the age of 70 years have mild calcification of the aortic valve, with over 10% having severe calcification [1]. Lastly, calcification of arterioles of the skin and other organs can lead to localized infarction and ischemia, including ischemic bowel and calciphylaxis [4].

Previously believed to be an end-stage process of unregulated mineral precipitation, cardiovascular calcification is now well established as a multi-faceted disease influenced by vascular location, the origin of calcifying cells and numerous regulatory pathways [2]. The pathogenesis of cardiovascular calcification is complex and involves transformation of vascular smooth muscle cells (VSMCs) to osteo/chondrocytic cells that express Runt-related transcription factor 2 (Runx2) and produce matrix vesicles. Imbalance of calcification promoters (such as BMP2, advanced glycation end-products (AGEs), hyperphosphatemia and hypercalcemia) and inhibitors (such as fetuin-A, matrix Gla protein and pyrophosphate) is critical for the development of cardiovascular calcification [4]. Furthermore, inflammation likely plays a significant role in its development. For example, results from *in vitro* studies and animal models of atherosclerosis suggest that inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-6 (IL-6) promote osteochondrogenic differentiation and vascular intimal calcification [5-7]. Inflammatory cytokines induce generation of reactive oxygen species (ROS), which have also been implicated in cardiovascular calcification [8]. Moreover, the pH of acidic cellular compartments also plays a role in osteo-/chondrogenic transformation and calcification of VSMCs [9].

High mobility group box 1 (HMGB1) is a nuclear constituent bound to chromatin in nearly all eukaryotic cells [10]. When released into the extracellular space upon cell activation, injury or death, HMGB1 functions as a damage-associated molecular pattern (DAMP) [11], and extracellular HMGB1 has drawn increasing attention with regard to its involvement in the pathogenesis of autoimmune and inflammatory diseases [12-14]. HMGB1 is also known as a bone-active cytokine that participates in both bone remodeling and ectopic calcification pathogenesis [15, 16]. Moreover, there is evidence that HMGB1 accumulates extracellularly in areas associated with macrophage infiltration and calcification in calcific aortic valve stenosis [17]. Wang et al. reported increased tissue and plasma levels of HMGB1 in patients with calcific aortic valve disease [18]. Our recent study demonstrated that HMGB1 induces matrix vesicle secretion by macrophages and leads to subsequent ectopic mineralization both *in vitro* and *in vivo* [19]. In addition, HMGB1 directly mediates VSMC osteoblastic differentiation in patients with diabetes [20].

However, studies about the roles of HMGB1 in promoting cardiovascular calcification are limited, and the underlying mechanisms remain unclear. In this review, we summarize recent studies on the mechanism of cardiovascular calcification and discuss the multiple roles of HMGB1 in the development of this condition.

The Basic Actions of HMGB1

In 1973, Goodwin discovered a group of nonhistone nuclear proteins characterized by high electrophoretic mobility and termed them high mobility group (HMG) proteins. These proteins include three superfamilies: HMGB, HMGN, and HMGA [21, 22]. HMGB1, also known as amphoterin, is a 30-kDa non-histone, chromatin-binding protein ubiquitously expressed in eukaryotic cells [23, 24]. The protein is 215 amino acids long and has a tripartite structure

consisting of two DNA-binding domains, an A box and a B box, and a C-terminal tail domain [25]. Constitutively expressed in most cell types, HMGB1 is predominantly located in the nucleus under physiological conditions, where it acts as a structural component in complex with chromatin and certain co-transcription factors. HMGB1 facilitates assembly of nuclear proteins and participates in DNA replication, recombination, transcription and repair [26, 27]. However, upon cell activation, injury or death, HMGB1 is translocated outside of the cell [9, 28]. Recent evidence reveals that the initial translocation of HMGB1 from the nucleus to the cytoplasm is regulated by JAK/STAT1-mediated acetylation [29], with subsequent extracellular release being partly controlled by dsRNA-activated protein kinase R (PKR)/inflammasome-mediated pyroptosis [30].

By binding to various receptors on the surface of immune competent cells, extracellular HMGB1 becomes a proinflammatory mediator serving as a DAMP or a so-called alarmin to stimulate the innate immune system [31-34]. In the extracellular environment, HMGB1 regulates inflammation as well as cell proliferation, survival and migration upon interaction with high-affinity receptors including receptor of advanced glycation end-products (RAGE), Toll-like receptors (TLRs) 2, -4, and -9, CXCR4, macrophage antigen-1, syndecan-3, CD24-Siglec-10, and T cell Ig mucin-3 [9, 21, 34]. The role of HMGB1 has been investigated in several systemic disorders, including sepsis, cancer, kidney diseases [36], thrombosis [37, 38], and certain autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [9, 33, 34]. HMGB1 is also a critical mediator in vascular diseases [39] such as atherosclerosis [35, 40, 41], myocardial ischemia/reperfusion (I/R) injury [42, 43], heart failure [44, 45], acute coronary syndrome (ACS) [46, 47], vasculitis [34], pulmonary artery disease [48-50], cerebral artery disease [51-53], and peripheral artery diseases (PADs) [54, 55].

Possible Mechanisms by Which HMGB1 Promotes Cardiovascular Calcification

Cardiovascular calcification is an age-related disease, particularly in association with atherosclerosis and diabetes mellitus [56]. It is currently acknowledged that aging is associated with a low-grade chronic inflammatory status, which can contribute to HMGB1 release [57]. Indeed, in atherosclerotic plaque and calcific aortic valve disease, HMGB1 is always found co-localized with calcification nidus [17]. Here, we review the literature on extracellular HMGB1 and its role in cardiovascular calcification. In addition, we present a schematic incorporating both the documented and speculated roles of HMGB1 in the pathogenesis of cardiovascular calcification.

HMGB1 and osteochondrogenic phenotype change

Several sources of cells that undergo osteochondrogenic differentiation in the vascular wall have been identified, including VSMCs, pericytes, adventitial myofibroblasts, interstitial valve cells, and circulating or stationary progenitor cells [2]. Aortic and valvular endothelial cells also have been shown to transition to cells that undergo osteochondrogenesis via the endothelial to mesenchymal transition (EMT) [58, 59]. Cells exhibiting an osteochondrogenic phenotype have the potential to generate mineralized matrix, resulting in calcified deposits [60]. Some studies have reported that HMGB1 participates in osteochondrogenic differentiation of certain cell types. Qi and his colleagues revealed that during human dental pulp cell (hDPC) odontoblastic differentiation, HMGB1 is translocated from the nucleus to the cytoplasm and then secreted into the extracellular space, promoting hDPC proliferation and mineralized nodule formation [61]. Similar results were observed in human periodontal ligament cells, which have been shown to undergo proliferation, migration, osteogenic differentiation, and biomineralization when exposed to HMGB1 [61]. Recently, it was demonstrated that HMGB1 can trigger the differentiation of mesenchymal stem cells into osteoblasts, which may provide a theoretical basis for improving clinical treatments for fractures [63, 64]. Wang et al. observed that HMGB1 promotes osteoblastic differentiation

and calcification of aortic valve interstitial cells (VICs) through TLR4-JNK-NF- κ B signaling [65].

Runx2, a key transcription factor of the Runx family, regulates osteoblast differentiation and chondrocyte maturation [66] and is an essential and sufficient regulator for inducing osteochondrogenic differentiation and calcification [66-68]. In normal vascular cells, expression of Runx2 is very low. However, Runx2 expression is significantly increased in calcified vascular tissue specimens from atherosclerotic plaque and arteries of chronic kidney disease (CKD) patients [68-71], suggesting an important role in cardiovascular calcification pathogenesis [72]. Runx2 influences cardiovascular calcification by regulating several osteogenic marker genes such as ALP, type I collagen, bone sialoprotein, and osteopontin [73]. HMGB1 can up-regulate the expression of RUNX2 and its downstream osteogenic markers such as alkaline phosphatase (ALP), osteopontin, and osteocalcin [62, 64]. These findings support the hypothesis that HMGB1 promotes cardiovascular calcification by inducing osteochondrogenic differentiation in multiple cells.

HMGB1 and TGF- β /BMP signaling

The transforming growth factor- β (TGF- β) superfamily of proteins contains cytokines and peptide growth factors that regulate biological functions in many systems, including heart valves and blood vessels [74]. TGF- β and its signaling pathways are strongly associated with fibrosis [75] and calcification [76], and previous studies show that TGF- β is characteristically present within calcified stenotic aortic cusps and mediates the calcification process of aortic valve interstitial cells in culture [77, 78]. Within the context of atherosclerotic calcification progression, TGF- β is abundantly expressed in VSMCs and promotes their osteogenic differentiation and calcifying potential [79]. A recent study by Krohn et al. showed that TGF- β induces the release of calcifying extracellular vesicles from VSMCs via the Smad3 and p38 pathways and regulates the vascular fibrocalcific response in atherosclerotic plaque [80]. Bone morphogenetic proteins (BMPs), which constitute a sub-group of the TGF- β superfamily, are potent activators of osteogenic differentiation and among the earliest factors described in the calcified artery wall [2].

Several interactions have been reported between HMGB1 and TGF- β or BMPs. For instance, Cheng et al. found that HMGB1 enhances AGE-induced expression of TGF- β in renal tubular epithelial cells via RAGE-dependent signaling [81]. HMGB1 was also recently shown to act through RAGE/NF- κ B/heparanase signaling to enhance active TGF- β release from the myofibroblast extracellular matrix (ECM) [82]. In addition, HMGB1 has an important role in mediating the calcification process induced by high glucose through BMP-2 expression in VSMCs of the saphenous vein [83]. These studies suggest that HMGB1 regulates the expression or release of TGF- β and BMP2 and may thus further promote cardiovascular calcification [84].

HMGB1 in secretion of calcific extracellular vesicles

Extracellular vesicles (e.g., microparticles, exosomes, matrix vesicles, apoptotic bodies) are membrane micro/nanovesicles secreted under physiological and pathological conditions by many cell types into the circulation and the extracellular milieu. Emerging evidence suggests that calcification may be initiated by the release of calcifying extracellular vesicles from cells residing in the calcification niche [80]. Matrix vesicles were initially found to be secreted from hypertrophic chondrocytes and osteoblasts during osteogenesis [85], initiating mineralization in two phases. First, in matrix vesicles, influx of calcium and phosphate via annexins and sodium-dependent inorganic phosphate transporters, respectively, leads to initial mineral accumulation in the form of hydroxyapatite crystals near the membrane. Second, mineral propagation in the ECM occurs, whereby hydroxyapatite crystals grow within the vesicles until the membrane ruptures; once exposed to the ECM, the crystals act as loci or templates for the formation of new crystals via homologous nucleation [85-88]. Alexander et al. observed that matrix vesicles released by VSMCs are also exosomal-like vesicles and that increased exosome release promotes cardiovascular calcification in

response to environmental calcium stress [89]. In addition, VSMC apoptosis often occurs in a several pathologic conditions and as a normal consequence of aging and stress. Apoptotic bodies derived from VSMCs have been shown to act as a nucleating structure for calcium crystal formation [90].

In our recent study, we demonstrated that HMGB1 can induce secretion of matrix vesicles by macrophages via the RAGE/p38/nSMase2 signaling pathway, and leads to subsequent mineralization *in vitro* and *in vivo* [19]. Moreover, HMGB1 was found to control the cellular apoptosis checkpoint during inflammation [91], hypoxia/reoxygenation injury [92] and hyperglycemia-induced apoptosis [93], which may be accompanied by release of a number of apoptotic bodies. All these findings support the hypothesis that HMGB1 promotes cardiovascular calcification by regulating the release of calcific extracellular vesicles.

HMGB1 in inflammation

Inflammation is likely to play a significant role in the development of cardiovascular calcification associated with atherosclerosis and diabetes mellitus, which has been confirmed in molecular imaging studies. Indeed, Abdelbaky et al. showed focal aortic inflammation, as detected by 18F-deoxyglucose positron emission tomographic scanning, in the aortic valve and atherosclerotic plaque to be significantly higher among patients who exhibited subsequent calcification progression [94]. Although cardiovascular calcification in the medial layer in association with CKD appears to link to long-term elevation of serum phosphate levels, apoptosis and uremic derangements [56], inflammation also acts in this process [95]. By releasing extracellular vesicles containing a phosphatidylserine-annexinV-S100A9 complex that facilitates mineral nucleation, macrophages are involved in the early, pro-inflammatory phase of calcification in atherosclerotic plaques [96]. Inflammatory cytokines, including TNF- α , IL-1 and IL-6, are associated with increased cardiovascular calcification [97, 98], and in a cohort free of clinically apparent cardiovascular disease, high C-reactive protein levels were associated with coronary artery calcification in both men and women [99]. Inflammatory cytokines such as TNF- α and IL-1 may exert their effects by stimulating the release of BMP-2 [100-102] or may enhance BMP-2 activity by reducing the levels of its inhibitor, matrix Gla protein (MGP) [97]. TNF- α also enhances matrix vesicle secretion by VSMCs [89].

HMGB1 is one of the endogenous mediators activating processes that lead to inflammation in the arterial wall. HMGB1 was also found to be associated with macrophage infiltration and areas of collagen accumulation and calcification [103]. Recombinant HMGB1 activates vascular endothelial cells, leading to expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and monocyte chemoattractant protein 1 (MCP-1), all of which contribute to leukocyte adherence and infiltration [104, 105]. Furthermore, HMGB1 promotes VSMC proliferation and osteochondrogenic differentiation and migration to the intimal layer in the development of atherosclerosis [41, 106], and growing evidence supports the role of inflammation in ECM remodeling, which drives the pathophysiology of cardiovascular calcification [1]. Moreover, HMGB1 is required for collagen deposition [106], ECM synthesis [108] and vessel remodeling [109, 110]. These observations suggest that HMGB1-induced inflammation participates in the initiation and propagation of cardiovascular calcification, particularly atherosclerotic calcification.

HMGB1 and Oxidative stress

In general, oxidative stress represents an imbalance between increased production of ROS and a reduction in the expression and activity of cellular antioxidant defense mechanisms. In the vascular wall, ROS are produced by several enzymes, including NADPH oxidase, xanthine oxidase, and uncoupled endothelial nitric oxide synthase (eNOS), as well as the mitochondrial electron transport chain. Nonetheless, the vasculature is protected by antioxidant enzymes, including superoxide dismutase (SOD), catalase, glutathione peroxidase and paraoxonase, which detoxify ROS. Cardiovascular risk factors such as hypercholesterolemia, hypertension, and diabetes mellitus enhance ROS generation, resulting in oxidative stress [111].

Fig. 1. Proposed roles of HMGB1 in cardiovascular calcification. HMGB1, high mobility group box 1; RAGE, receptor for advanced glycation end products; nSMase2, neutral sphingomyelinase2; TGF- β , transforming growth factor- β ; BMP, bone morphogenetic protein; ROS, reactive oxygen species; EVs, extracellular vesicles; VSMCs, vascular smooth muscle cells; IVC, interstitial valve cell; Runx2, runt-related transcription factor 2; MSX2, Msh homeobox 2; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; BSP, bone sialoprotein.

Increased levels of ROS and oxidative stress have been shown to participate in the pathogenesis of cardiovascular calcification [112]. Heightened ROS generation has been predominately found around calcifying foci, potentiating aortic valve calcification progression in rabbits [113]. Furthermore, ROS, especially superoxide produced by NADPH oxidase, mediate osteoblastic differentiation, ECM disorganization and cardiovascular calcification induced by uremic serum [114] or β -glycerophosphate [115], BMP-2 [116], AGEs [117, 118] and the proinflammatory cytokine TNF- α [119, 120]. In addition, much evidence suggests that antioxidants might have potential benefits in the management of cardiovascular calcification. For example, vitamin E was found to antagonize acceleration of cardiovascular calcification in hypercholesterolemia-induced rats [121], and the antioxidant TEMPOL, a SOD mimetic, ameliorated osteoblastic differentiation of VSMCs and arterial medial calcification in uremic rats [122]. Together, these data confirm that oxidative stress plays a critical role in the pathogenesis of cardiovascular calcification.

Previous studies have indicated that oxidative stress can regulate HMGB1 release in various cells [123] and mediate differentiation induced by ROS [124]. HMGB1 has also been shown to enhance ROS via a positive feedback loop in the apoptotic process of diabetic retinopathy [125] and diabetes-induced endothelial progenitor cell dysfunction [126]. Through TLR4-ROS signaling, HMGB1 impairs cardiac excitation-contraction coupling by enhancing sarcoplasmic reticulum Ca^{2+} leakage in cardiomyocytes [127]. Taken together, mutual enhancement between HMGB1 and oxidative stress may further promote cardiovascular calcification, and the mechanisms underlying this process are possibly involved in osteochondrogenic differentiation and apoptosis.

HMGB1 and Autophagy

Homeostasis and rapid adaptation to environmental changes are vital to organismal health and survival. Autophagy is an evolutionarily conserved process by which long-lived proteins and organelles are sequestered by autophagosomes and subsequently degraded by lysosomes for recycling [128]. Growing evidence reveals that basal autophagy is essential to the process that mediates proper vascular function. Moreover, autophagy is induced by many stress-related stimuli in the vascular wall to protect VSMCs and endothelial cells against cell death and the initiation of vascular diseases such as atherosclerosis, aneurysm formation, arterial aging, vascular stiffness, and chronic venous disease [129, 130]. It

appears that autophagy affects cardiovascular calcification. However, most of the work thus far has focused on the role of autophagy in osteoblastic differentiation. One study reported that autophagy is involved in the osteogenic differentiation of odontoblasts [131] and human osteoblast associated with the titanium-based dental implants [132]. In mesenchymal stem cells (MSCs), autophagy is induced by forkhead box O3 (FOXO3) to reduce ROS resulting from the increased mitochondrial respiration that occurs during osteoblast differentiation [133] and may regulate osteogenic differentiation via AMPK/Akt/mTOR signaling [134]. VSMCs stimulated with platelet-derived growth factor (PDGF) show autophagy induction, which regulates a synthetic phenotypic transition in response to oxidative stress [135]. Furthermore, Dai et al. recently described that VSMCs treated with high phosphate exhibit upregulation of autophagic markers such as LC3 and autophagosomes in the cytoplasm, which can counteract cardiovascular calcification by reducing matrix vesicle release [136]. In addition, TGF- β 1-stimulated calcification of VSMCs can be alleviated by atorvastatin-induced autophagy [137], which also indicates the important role of autophagy in regulating cardiovascular calcification.

Accumulating data suggest that HMGB1 is an important autophagy regulator during cell stress. A recent study from Zhu et al. reports that cytosolic HMGB1 controls the cellular autophagy/apoptosis checkpoint during inflammation [91]. In addition, HMGB1-mediated autophagy regulates the differentiation of acute promyelocytic leukemia cells in response to oxidative stress [124]. Given the role of autophagy in cardiovascular calcification, it appears reasonable that HMGB1 may strongly influence cardiovascular calcification by regulating autophagy.

Conclusions

The possible mechanisms of HMGB1 in cardiovascular calcification are depicted in Fig. 1. Although evidence suggesting the direct involvement of HMGB1 in cardiovascular calcification is limited, an increasing number of studies have shown that both extracellular and cytosolic HMGB1 have a series of pro-calcification effects, such as promoting osteochondrogenic differentiation, apoptosis and release of calcific extracellular vesicles via TGF- β /BMP, inflammation, oxidative stress and autophagy signaling. These results highlight the fact that inhibition of HMGB1 may be a potential therapy for attenuating cardiovascular calcification. Regardless, the mechanisms by which HMGB1 promotes cardiovascular calcification have not been fully elucidated. Thus, future studies should pay particular attention to exploring these mechanisms in detail. Recently, some approaches have been shown to be protective in models of vascular pathologies at least in part, via inhibiting HMGB1 expression, release, or activity [39, 138-140]. With more clues uncovered, other approaches to preventing HMGB1 release or binding to its receptors will provide novel strategies for treating cardiovascular calcification and reducing cardiovascular events. However, as HMGB1 also functions as a bone-active cytokine [15], future studies also should notice the impact of inhibiting HMGB1 on skeletal calcification.

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Disclosure Statement

The authors declare no conflict of interest.

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