Cardiovascular calcification is an independent risk factor for cardiovascular morbidity and mortality. This disease of dysregulated metabolism is no longer viewed as a passive degenerative disease, but instead as an active process triggered by pro-inflammatory cues. Furthermore, a positive feedback loop of calcification and inflammation is hypothesized to drive disease progression in arterial calcification. Both calcific aortic valve disease and atherosclerotic arterial calcification may possess similar underlying mechanisms. Early histopathological studies first highlighted the contribution of inflammation to cardiovascular calcification by demonstrating the accumulation of macrophages and T lymphocytes in 'early' lesions within the aortic valves and arteries. A series of in vitro work followed, which gave a mechanistic insight into the stimulation of smooth muscle cells to undergo osteogenic differentiation and mineralization. The emergence of novel technology, in the form of animal models and more recently molecular imaging, has enabled accelerated progression of this field, by providing strong evidence regarding the concept of this disorder as an inflammatory disease. Although there are still gaps in our knowledge of the mechanisms behind this disorder, this review discusses the various studies that have helped form the concept of the inflammation-dependent cardiovascular calcification paradigm.  

Key Words: Aortic valve; Atherosclerosis; Calcification; Inflammation; Molecular imaging

Westernized countries face a growing burden of cardiovascular calcification, a disease of dysregulated mineral metabolism that leads to increased acute cardiovascular events and potentially death. A number of risk factors are known to accelerate atherosclerosis and cardiovascular calcification, including increased age, hypercholesterolemia, metabolic syndrome, endstage renal disease and diabetes mellitus. Cardiovascular calcification results in the deposition of calcium, principally in the form of hydroxyapatite, in the vessel wall and the leaflets of the aortic valve. Mounting evidence suggests that the process of cardiovascular calcification involves similar mechanisms to that of bone development, such as osteoblastic differentiation and mineralization. This disorder was once regarded as a passive degenerative disease, but it is now widely recognized as an active process associated with inflammation.

Cardiovascular calcification was previously thought of just as an indicator of increased cardiovascular events; however, it is now apparent that this disorder also contributes to cardiovascular risk. Arterial calcification causes a reduction in the elasticity of the vessel wall and thus reduced compliance, and in certain situations can lead to fatal myocardial infarction. In addition, calcification of the aortic valve is a progressive disease that impairs the movement of the aortic valve leaflets, leading to valve dysfunction, left ventricular remodeling and even heart failure. Despite its vast clinical significance the mechanisms of ectopic calcification remain unclear and no therapies are available to prevent disease progression. The National Heart, Lung and Blood Institute (NHLBI) Working Group on Calcific Aortic Stenosis has recently emphasized the critical importance of defining novel calcification mechanisms and the need to develop new animal models and imaging modalities for detection of subclinical calcification.

Conventional diagnostic imaging techniques, including coronary angiography, computed tomography and transthoracic echocardiography, are proficient at visualizing anatomic changes associated with cardiovascular calcification. At present surgical intervention is the only means to treat this disease; minimally invasive angioplasty procedures or invasive bypass surgery are effective ways of treating calcified arteries in the short term, and surgical aortic valve replacement with a range of substitutes is the only effective treatment of calcific valve disease. Various therapeutic agents have been or are being investigated to target cardiovascular calcification and thus improve quality of life for patients; these include statins, mineralocorticoid receptor antagonists and bisphosphonates; however, as yet they have not proved beneficial in the clinical setting. The emergence of novel technology, in the form of animal models and more recently molecular imaging, has enabled accelerated progression of this field, in particular, providing strong evidence regarding the concept of cardiovascular calcification as an inflammatory disease.
Calcific Aortic Valve Disease (CAVD)

CAVD is a progressive disorder that ranges from early alterations in leaflet cell biology to advanced calcification characterized by calcific lesions on the aortic surface of the valve cusp, resulting in impaired movement of the aortic valve leaflets followed by left ventricular outflow obstruction. Our increasingly aged and dysmetabolic population is experiencing an epidemic of CAVD, which is becoming a growing concern. However, although CAVD is more common with age and for decades was thought to be a passive degenerative process, it is not an inevitable consequence of aging. Indeed, emerging studies on calcified aortic valves have provided evidence that this disease involves an actively regulated process that bears similarities to endochondral bone formation. Furthermore, clinicopathologic studies found that early lesions contain inflammatory cells, which has been corroborated in vivo using a hypercholesterolemic rabbit model and more recently in mouse models of aortic valve calcification. These studies have not only indicated that the underlying mechanisms of CAVD are similar to that of atherosclerotic arterial calcification, but have also established the concept of inflammation-dependent development of aortic valve calcification.

Atherogenic factors and/or mechanical stress activate valvular endothelial cells, thus causing them to express adhesion molecules. This was demonstrated in our molecular imaging study, which simultaneously visualized in vivo key cellular events in aortic valve calcification. (Adapted from Aikawa E et al with permission.)

Figure 1. Visualizing aortic valve calcification. (A) Magnetic resonance imaging (MRI) can detect early valvular dysfunction in aortic valve disease in vivo; the regurgitation jet causes the typical black signal (arrow). (B) Ex vivo MRI. Endothelial cell activation occurs in the commissures of diseased aortic valves. (Upper) Long-axis view demonstrates the aortic arch and root. Dotted line depicts slice position of short-axis view. (Lower) Color-coded signal intensities of short-axis view show focused uptake of VCAM-1 (red) in commissures (arrows). (C) Thickened valve leaflets noted in hypercholesterolemic apoE−/− mice associated with accumulation of macrophages (arrows). H&E. Original magnification ×100. (D) Valvular myofibroblasts exhibit osteoblastic transition as detected by Runx2/Cbfa1 (red reaction product) in the early stage of aortic valve stenosis. Original magnification ×400. (E) Gross morphology of calcified aortic valves. (F) Ex vivo fluorescence microscopy (image stacks) of calcified aortic valves visualizes the osteogenic activity (red) and macrophage activity (green) that overlaps in the areas of leaflet attachment to the aortic wall in inflamed valves. VCAM, vascular cell adhesion molecule. (Adapted from Aikawa E et al with permission.)
Cardiovascular Calcification

Mauro et al. previously demonstrated that endothelial cell activation, macrophage accumulation, expression of matrix remodeling enzymes, and osteogenic activity. In our study, endothelial VCAM-1 expression was found adjacent to an area in the aortic valve leaflets that is known to encounter the greatest amount of mechanical stress. As observed in multiple studies the activation of endothelial cells results in the expression of adhesion molecules and subsequent recruitment of monocytes and macrophage accumulation in the extracellular matrix (ECM) of the valve. In addition, a recent study has demonstrated that mechanical stress may induce the osteogenic potential of valvular endothelial cells, providing evidence that the valvular endothelium harbors a reserve of progenitor cells that can repopulate the leaflet with osteogenic-like interstitial cells. Future studies are needed to show whether this mechanism, in combination with pro-inflammatory cues, contributes to pathological valve calcification.

A variety of studies have demonstrated that macrophages and “activated” valvular interstitial cells (also known as myofibroblasts) elaborate excessive levels of proteolytic activity, in the form of matrix metalloproteases (MMPs) and cysteine endoproteases, which degrade collagen and elastin in the valvular ECM. Kaden et al. demonstrated in vitro that pro-inflammatory cytokines expressed in stenotic aortic valves, including interleukin (IL)-1, tumor necrosis factor (TNF) α and RANKL, regulate remodeling of the ECM by stimulating myofibroblasts to produce MMPs. ECM remodeling and thickening of the leaflets due to the action of matrix proteases.

Figure 2. Intravital fluorescence microscopy monitoring of changes in inflammation and osteogenesis in mouse carotid arteries. (A) At 20 weeks, mice were randomized either to continue with the high-cholesterol diet or to consume the high-cholesterol diet admixed with a statin for 10 more weeks. Intravital microscopy was performed sequentially at 20 and 30 weeks, and at 30 weeks on the statin diet. Image stacks simultaneously visualized 2 different biological processes: inflammation (green) and osteogenesis (red). (B) Quantification demonstrated that the macrophage- and osteoblast-derived near-infrared signal intensities increased over time and decreased after statin treatment. AU, arbitrary units. (Adapted from Aikawa E et al* with permission.)
remodeling enzymes may result in valvular dysfunction and further alteration of mechanical stresses across the valve leaflet.

The valvular interstitial cells possess a certain amount of plasticity, interchanging between quiescent fibroblast-like cells and the “activated” myofibroblasts. In addition valvular interstitial cells are able to become osteoblast-like cells in vitro, differentiating into osteoblastic cells through augmentation of the Runx2/Cbfa1 and Notch1 pathways. The remodeling of the valve leaflet’s ECM due to proteolytic activity is hypothesized to result in valvular dysfunction and an alteration of the mechanical stresses across the valve leaflet, which may further induce inflammation and trigger osteoblastic differentiation. The end result would be deposition of calcium primarily in the regions of high mechanical stress and eventual mobilization of the aortic leaflets due to increased valve stiffness (Figure 1).

**Arterial Calcification**

Arterial calcification, which occurs as part of the atherosclerotic process, has been recognized for over 200 years; however, it was only at the end of the 20th century that this field was rediscovered. For the past decade the emphasis of studies has been on the active nature of this disorder. An epidemiological study highlighted the significance of dysregulated mineral metabolism in the acceleration of vascular calcification, while spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein (MGP) indicated that ECM calcification is actively inhibited in soft tissues by MGP. The mechanism of arterial calcification has been suggested to follow an endochondral bone formation process, in which the osteoblastic differentiation of vascular smooth muscle cells (SMCs) is via the Cbfa1 pathway, akin to the bone osteoblastic differentiation and similar to valvular interstitial cells as described previously. This process is associated with the expression of a number of transcription factors associated with osteoblasts and chondrocytes (eg, Cbfa1, Osterix, Sox9), and mineral-regulating proteins (eg, MGP, alkaline phosphatase, osteocalcin and osteopontin) associated with SMCs. Furthermore, a number of recent studies have contributed to the concept that arterial calcification is a progressive pro-inflammatory disorder.

Monocytes and macrophages have been observed to enhance vascular calcification in vitro via both cell–cell interaction and the production of soluble factors. A series of studies established that inflammatory cytokines such as IL-1β, IL-6, IL-8, insulin-like growth factor-1 and TNFα induce osteogenic differentiation and mineralization of vascular cells in vitro, suggesting that inflammatory cytokines initiate or promote cardiovascular calcification by regulating the differentiation of calcifying vascular cells.

Our molecular imaging studies on arterial calcification not only provided the first in vivo evidence of the role that inflammation plays in initiating calcification, but also for the first time demonstrated the dynamic osteogenic changes during the progression and regression of atherosclerotic plaques. To monitor in vivo osteogenic changes we performed sequential intravital microscopy on the carotid arteries of untreated and statin-treated cohort of mice at 20 weeks and 30 weeks of age (Figure 2). Macrophage numbers were found to increase in association with osteogenic activity at 30 weeks; however, both the macrophage burden and osteogenic activity were prevented by anti-inflammatory statin therapy. In addition, while observing the disease progression in living mice, we observed that both inflammation and calcification progressed quickly from 20 to 30 weeks: the overall volume of macrophages increased up to 160% while calcification increased over 25% (Figure 3). This study, along with other robust evidence, has enabled us to further elaborate on the
Inflammation-dependent calcification paradigm. This paradigm can be split into 3 stages: initiation, propagation and endstage calcification.\textsuperscript{5,49}

In the initiation stage, macrophages precede calcification while releasing pro-osteogenic cytokines. In the propagation stage, the stimulated vascular SMCs undergo osteogenic differentiation or release of calcifying matrix vesicles. In addition, dying cells undergo apoptosis and release apoptotic bodies. Both the calcifying matrix vesicles and apoptotic bodies may provide new foci for hydroxyapatite nucleation, resulting in microcalcifications. During propagation, both inflammation and microcalcifications develop within close proximity, overlap at the border regions and can be observed simultaneously (Figure 4).\textsuperscript{5} The final stage (ie, endstage calcification) is associated with little to nil inflammation and advanced tissue mineralization. It is accepted as more difficult to treat this stage of calcification, and it is classically reviewed as irreversible. Therefore, it is hypothesized that utilizing anti-inflammatory therapy, perhaps in the form of statins, at the earlier stages would prove beneficial in reducing inflammation and halting subsequent osteogenesis.\textsuperscript{5,16}

To date, clinical trials have not demonstrated the benefit of statin therapy in halting valve calcification progression;\textsuperscript{11} however, this may be due to the late implementation of the treatment when the calcification had advanced to the later stages.

Role of Matrix Vesicles and Microcalcifications in Plaque Rupture

Due to the role that matrix vesicles play in physiological mineralization of bone and cartilage,\textsuperscript{50} it is suggested that they also play a sizeable role in pathological ectopic calcification; in fact, matrix vesicles are thought to predominate in aortic calcification.\textsuperscript{51} In atherosclerotic calcification, macrophages elaborate pro-osteogenic cytokines that stimulate vascular SMCs to release calcifying matrix vesicles (30–300 nm), which bud from the plasma membrane of cells. Therefore, although the precise role that matrix vesicles play in arterial calcification and the mechanism by which they are released still require further investigation, it is believed that they serve as a nidus for mineral nucleation and are thus associated with the generation of microcalcifications in the propagation stage of the calcification paradigm.\textsuperscript{50} Furthermore, matrix vesicles contain inhibitors of mineralization, such as MGP and fetuin-A, and a reduction in these inhibitors enhances tissue mineralization.\textsuperscript{52} Serum fetuin-A expression has been shown to be downregulated by inflammation,\textsuperscript{53} suggesting that inflammation may act in this form of calcification by downregulating inhibitors of mineralization and thereby enhancing mineralization of the vesicles.

The generation of microcalcifications occurs in the presence of inflammation, and lesions in this early stage of the calcification paradigm are often described in imaging studies as “spotty” calcifications.\textsuperscript{54} This stage is therefore associated with an inflammation-dependent progression of calcification, in contrast to advanced calcific plaques. Indeed more advanced calcification has been suggested as more stable due to negligible inflammation.\textsuperscript{55} In vitro studies by Nadra et al have demonstrated that microcalcifications induce a pro-inflammatory response in macrophages via mechanisms involving protein kinase C-α and ERK1/2 MAP kinase.\textsuperscript{56,57} These in vitro studies were the first to suggest that a positive feedback loop of calcification and inflammation may drive progression of arterial calcification, which was corroborated by our in vivo molecular imaging studies.\textsuperscript{5,58} Microcalcifications located in the thin (<65 μm) fibrous cap overlying the necrotic core of atherosclerotic plaques are seen as dangerous, as they are more likely to cause plaque rupture due to debonding\textsuperscript{59} and lead to acute thrombosis and sudden death.
due to fatal myocardial infarction. A summary of macrophage-triggered formation of microcalcifications via matrix vesicle release can be seen in Figure 5.

**Role of Inflammation in Calcification Associated With Chronic Renal Disease (CRD) and Diabetes**

Clinical studies have demonstrated that 50% of patients with CRD die from a cardiovascular disease and this is increased to 80% for patients with diabetes mellitus, a major cause of CRD. The inflammatory cytokine, TNFα, and inflammation-induced oxidative stress have been demonstrated to contribute to arterial calcification associated with diabetes by promoting pro-calcific Msx2-Wnt signaling cascades. In addition, arterial calcification associated with CRD, which accompanies diabetes, occurs in response to altered calcium and phosphate metabolism and has been demonstrated to be the result of an imbalance of inhibitors and inducers of mineralization. Both the intima and media of the arteries have been observed to calcify in patients with these disorders. Medial calcification has been noted to occur without the infiltration of macrophages. However, preclinical and clinical evidence suggests that CRD promotes a pro-inflammatory milieu and accelerates the development of atherosclerosis and intimal calcification.

Calcification associated with diabetes and CRD has been suggested to be initiated via matrix vesicle-nucleated mineralization, as demonstrated in both atherosclerotic calcification and CAVD. Elevated extracellular calcium and phosphate levels induce osteogenic differentiation of vascular SMCs and release of calcifying matrix vesicles. In addition, vesicles have been observed in the calcifying arteries of uremic patients. Calcification mediated by vascular smooth muscle cell-derived matrix vesicles is regulated by both inhibitors and promoters of calcification, so it has been suggested that cardiovascular calcification associated with CRD and diabetes is a protective response to inflammation. Furthermore, inflammation may induce elastases, such as cathepsin S, to degrade elastic fibers, thus producing elastin-derived peptides that promote the osteogenic differentiation of SMCs and subsequent calcification.
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Role of Inflammation in Cardiovascular Calcification and Osteoporotic Bone Remodeling

Studies in the 1990s declared an association among osteoporosis, cardiovascular calcification, atherosclerosis and CRD. A reduction in bone mineral density (BMD) was noted with the progression of cardiovascular disease, particularly in women. Demer and Tintut mused in a recent review that this paradox is a tissue-specific responses to chronic inflammation. Our study, utilizing both 3D micro-CT and optical molecular imaging, demonstrated that both bone osteogenic activity and BMD decrease as cardiovascular calcification develops; indeed, the degree of cardiovascular calcification directly correlated with the loss of BMD. There are various schools of thought as to how this paradox occurs: for example, some have suggested that osteoporosis causes the release of osteogenesis- and mineralization-promoting biochemical factors, which induce the cardiovascular system to calcify. However, despite the exact mechanism requiring further elucidation, the evidence produced in our molecular imaging study unequivocally demonstrated that systemic and local inflammation paradoxically drives both cardiovascular calcification and bone loss (Figure 6).

Conclusion

Over the past decade a multitude of studies have provided compelling evidence that cardiovascular calcification is an inflammatory process. Accumulating evidence suggests that calcific aortic valve disease and arterial calcification have similar underlying mechanisms, stemming from the infiltration and accumulation of monocytes/macrophages. The extent to which inflammation plays a role in arterial calcification associated with CRD and diabetes still needs to be discovered. Furthermore, the concept of an inflammation-dependent pathway has been established for both of those disorders. In the same manner, although it has been established that inflammation drives progression of both osteoporosis and cardiovascular calcification, the underlying mechanism still needs to be elucidated. Our studies have demonstrated the benefits of molecular imaging modalities to investigate cardiovascular calcification. Molecular imaging could be used to not only elucidate the mechanisms of calcification in vivo, but also has the potential to be a diagnostic tool and predict the risk of subclinical calcific lesions. The clinical feasibility of this modality is becoming clear: agents for use in optical molecular imaging are being considered for clinical trials and technology is being developed to use this modality clinically. The use of novel technology alongside original in vitro mechanistic studies will allow us to build on what is already known about the role that inflammation plays in calcification. Cardiovascular calcification is an ever-increasing problem, thus the necessity to enhance research into this field is crucial to yield potential therapeutic targets and therefore improve the outcome for patients.

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