Molecular Imaging Insights Into Early Inflammatory Stages of Arterial and Aortic Valve Calcification

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Abstract: Traditional imaging modalities such as computed tomography, although perfectly adept at identifying and quantifying advanced calcification, cannot detect the early stages of this disorder and offer limited insight into the mechanisms of mineral dysregulation. This review presents optical molecular imaging as a promising tool that simultaneously detects pathobiological processes associated with inflammation and early stages of calcification in vivo at the (sub)cellular levels. Research into treatment of cardiovascular calcification is lacking, as shown by clinical trials that have failed to demonstrate the reduction of calcific aortic stenosis. Hence, the need to elucidate the pathways that contribute to cardiovascular calcification and to develop new therapeutic strategies to prevent or reverse calcification has driven investigations into the use of molecular imaging. This review discusses studies that have used molecular imaging methods to advance knowledge of cardiovascular calcification, focusing in particular on the inflammation-dependent mechanisms of arterial and aortic valve calcification. (Circ Res. 2011;108:1381-1391.)

Key Words: aortic valve ■ atherosclerosis ■ inflammation ■ calcification ■ molecular imaging

Cardiovascular calcification, a disease of dysregulated mineral metabolism, is by no means a new dilemma. Indeed, some reports date its existence as far back as the Ice Age. Hypercholesterolemia, metabolic syndrome, end-stage renal disease, diabetes mellitus, and increased age accelerate atherosclerosis and cardiovascular calcification. Ectopic mineralization mainly affects the aorta, coronary arteries, peripheral arteries, and aortic valves, with fully formed bone observed in atherosclerotic plaques and stenotic aortic valves. Once believed to be a passive degenerative disease, cardiovascular calcification is now recognized as an active process, with evidence suggesting that it follows a mecha-
nism similar to that of bone formation. Age and lifestyle are still major factors, however, and thus, the rising average age of the population is accompanied by a growing burden of this disorder, which translates into a large cost for society.5–6

**Cardiovascular Calcification: An Unresolved Medical Problem**

**Arterial Calcification**

Cardiovascular calcification, typically measured and quantified in patients using imaging modalities such as computed tomography (CT), serves as a marker for atherosclerotic coronary artery disease and is associated with increased cardiovascular events. Coronary artery calcification scoring produced via the use of CT has been shown to predict future coronary heart disease events. Arterial microcalcifications located in the thin (<65 μm) fibrous cap overlying the necrotic core of atherosclerotic plaques may cause microfractures, which can lead to acute thrombosis and even fatal myocardial infarction. However, although evidence suggests that microcalcifications in thin fibrous caps increase the risk of plaque rupture, calcification remains a neglected pathology, and effective anti-calciﬁcation therapies are not available.

**Aortic Valve Calcification**

Massing evidence suggests that valvular calcification possesses characteristics of arterial calcification. Clinico-pathological studies of human stenotic aortic valves have identiﬁed lesions similar to those in atherosclerotic plaques, and atherosclerotic-like lesions have been noted in the aortic valve leaflets of rabbit and mouse models of atherosclerosis. Aortic valve stenosis and coronary atherosclerosis also share epidemiological risk factors, which further fuels recognition of their similarity. Calcific aortic valve disease can range from mild valve thickening to severe calcification with impaired leaflet motion or aortic valve stenosis, the most common form of heart valve disease. Thus, calcification is a strong predictor of disease progression in patients with initially asymptomatic aortic stenosis. Approximately 85,000 patients in the United States and 275,000 worldwide annually undergo valve replacement because of aortic valve stenosis; invasive and costly surgical intervention is the only effective treatment.

**Current Treatment Strategies**

Current research is aimed at revealing the mechanisms involved in cardiovascular calcification so that specific pathways can be targeted pharmacologically. Various therapeutic agents have been investigated, including statins, which have decreased osteoblastic differentiation and cell mineralization in vitro and prevented progression of macrophage burden and osteogenesis in vivo. However, thus far, statin therapy has not proved beneﬁcial in clinical trials. Because no therapies are currently available to prevent or treat calcific disease progression, the Working Group on Calcific Aortic Stenosis of the National Heart, Lung, and Blood Institute (NHLBI) recently highlighted the importance of developing new imaging and therapeutic strategies to diagnose, prevent, and potentially reverse or delay the onset of the calcification process. The present review aims to establish underlying mechanisms of cardiovascular calcification as elucidated by in vivo molecular imaging and demonstrate the prognostic value of optical near-infrared fluorescence (NIRF) imaging for the detection of speciﬁc changes associated with arterial calcification and aortic valve disease.

**The Evolving Molecular Imaging Approach**

**Conventional Imaging Modalities for Detection of Calcification**

The detection and quantiﬁcation of advanced calcification in coronary arteries and aortic valves can be readily achieved by conventional diagnostic imaging techniques, including CT, intravascular ultrasonography (IVUS), transthoracic echocardiography, and magnetic resonance imaging (MRI). At present, IVUS is an excellent tool for detecting advanced calcification; however, calcium volume is hard to quantify with this method because of acoustic shadowing. It is clinically advantageous for diagnostic imaging techniques to be noninvasive as possible to alleviate patient discomfort. Therefore, noninvasive techniques such as CT and ultrasound are emerging as diagnostic contenders, particularly because they are more sensitive than other imaging modalities and can quantify calcium content. However, most imaging modalities have low spatial resolution and lack suitable molecular imaging agents and thus cannot detect the earliest stages of calcification/osteogenesis on cellular and molecular levels.

**Optical Molecular Imaging**

Although conventional imaging modalities profi ciently visualize anatomic structures and macroscopic changes, high-resolution optical imaging, particularly intravital fluorescence microscopy, enables observation of processes on molecular and (sub)cellular levels. Novel molecular imaging technologies use targeted and activatable imaging agents for the in vivo detection of proinflammatory, pro-osteogenic, and proteolytic activity. Chen et al demonstrated the potential use of proteases as biomarkers for vulnerable plaques when probed with beacons; we have since harnessed this technique to explore the proinﬂammatory mechanisms of cardiovascular calcification. Imaging agents use speciﬁc molecular or cellular processes to generate image contrast with high-resolution imaging technology. Advances in nanotechnology have yielded targeted imaging agents by chemical attachment of an afﬁnity ligand, such as an antibody or small molecule, to a ﬂuorochrome or magnetic compound (eg, bisphosphonate-conjugated ﬂuorescent agent for the detection of hydroxyapatite or cross-linked iron oxide ﬂuorescent nanoparticles for the detection of macro
phages\textsuperscript{23,34,38}). Studies have also produced activatable imaging agents—chemically engineered substrates that interact with their targets (eg, enzymes) and undergo a physicochemical change, which results in signal amplification (eg, protease-activatable imaging agents for the detection of matrix metalloproteinase or cathepsin activity).\textsuperscript{23,39,41,46}

NIRF (excitation in the 650- to 900-nm wavelength) molecular imaging represents a useful platform for optical molecular imaging in vivo.\textsuperscript{47,48} Indeed, near-infrared light has the potential to penetrate tissues in the magnitude of centimeters rather than micrometers,\textsuperscript{49} which makes NIRF imaging highly attractive, because it allows for greater depth sensing of a larger area of inflamed tissue or calcific lesion. In addition, fluorescence imaging in the near-infrared bandwidth offers reduced tissue autofluorescence.\textsuperscript{48} Other imaging detection systems can also be used, depending on the resolution required. One such platform is fluorescence-mediated tomography, which can detect femtomole quantities of fluorochromes in whole animals with millimeter resolution.\textsuperscript{39,40}

Fluorescence molecular imaging is able to use 2 or more spectrally distinct imaging agents to visualize different biological processes simultaneously.\textsuperscript{23,34,41,42,50} It can also be integrated with more conventional imaging techniques (eg, MRI and CT) when used with multimodality or multifunctional probes. A recently developed trimodality iron oxide–based magnetic nanoparticle could be used for simultaneous NIRF, MRI, and positron emission tomography imaging of macrophages.\textsuperscript{51} Therefore, it may enable the attainment of both high imaging sensitivity (from NIRF) and high spatial resolution (from MRI).\textsuperscript{52}

Clinical Translation

For the successful treatment of calcification, we need to visualize the pathways involved in the earlier stages; studies that apply optical molecular imaging to the calcifying vasculopathy/valvulopathy or bone remodeling are therefore desired. We anticipate that clinical molecular imaging approaches could provide new biological insights into human arterial osteogenesis far before the development of advanced calcification detected by current methods. To accomplish this goal, clinical multimodal molecular imaging approaches will likely be required to detect and monitor the dynamic changes in inflammation/macrophages and osteogenesis/calcification in calcified arterial valves and atherosclerotic plaques in cardiovascular calcification.

Studies to improve molecular imaging methods and increase their chances for clinical use are ongoing. Clinical molecular imaging has already made significant headway into visualizing targets in large vessels.\textsuperscript{53} There is substantial evidence supporting the use of \textsuperscript{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) imaging for the evaluation of coronary artery disease.\textsuperscript{54} Furthermore, a novel intravascular NIRF catheter has been developed that has been demonstrated to detect inflammation-associated protease activity in vessels the size of human coronary arteries in real time with an activatable NIRF agent.\textsuperscript{55} In addition, more recently, a new 2-dimensional NIRF imaging catheter system based on rotational fiber design has been developed that will allow seamless integration of molecular imaging into the cardiac catheterization laboratory.\textsuperscript{56} These advanced molecular imaging techniques not only offer the potential to be sensitive diagnostic tools, they also enable in vivo study of the mechanisms of atherosclerosis and cardiovascular calcification. For example, the availability of a clinical intravascular NIRF catheter could accelerate the detection of high-risk plaques.\textsuperscript{53} Because of the significant technological developments made in the field of molecular imaging over the past 2 decades, it is now deemed a clinically feasible diagnostic tool.\textsuperscript{52}

Imaging Identifies Underlying Molecular Mechanisms Involved in Early Aortic Valve Calcification

The mechanistic pathways involved in the development of calcific aortic valve disease remain largely unknown. Therefore, the use of molecular imaging is considered advantageous to detect early molecular and functional abnormalities in aortic valves. Our recent research tested the hypothesis that molecular imaging can detect early changes in aortic valve disease, with positive outcomes.\textsuperscript{23} We used a panel of distinct NIRF imaging agents to map endothelial cells, macrophages, proteolytic activity, and osteogenesis in the aortic valves of hypercholesterolemic apolipoprotein E–deficient (apoE\textsuperscript{−/−}) mice.

Imaging of Valvular Endothelial Cell Activation

MRI and NIRF microscopy clearly demonstrated ex vivo that most of the vascular cell adhesion molecule-1–targeted agent\textsuperscript{57} was distributed in the aortic valve leaflets near the attachment of the aortic root (a region known as the commissures), which was corroborated by immunohistochemical evidence with immunoreactive vascular cell adhesion molecule-1.\textsuperscript{23} Increased expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin has been noted in surgically removed diseased heart valves,\textsuperscript{58,59} which illustrates that injury to endothelial cells causes increased expression of adhesion molecules. The leaflets of the heart valves open and close at least 3×10\textsuperscript{9} times over a single lifetime; therefore, they have to endure a certain amount of “wear and tear” due to the repetitive forces exerted on them. During the cardiac cycle, the aortic valve leaflets are subjected to continual bending, shearing, and tensile and compressive stresses.\textsuperscript{60} Because the flexion areas of the aortic valve leaflets near the commissures encounter the greatest amount of mechanical stress within the leaflet,\textsuperscript{61} these areas might induce endothelial cell activation/injury and thus the subsequent expression of adhesion molecules.

Imaging of Macrophages and Proteolytic Activity

Elevated plasma lipids and other atherogenic factors may also induce valve endothelial cell activation. This results in an amplification cascade of events, including monocyte recruitment and subsequent macrophage accumulation within the extracellular matrix of the valve, as visualized by macrophage-targeted, NIRF-conjugated iron nanoparticles. The use of NIRF protease-activatable probes provided direct evidence that valvular interstitial cells (in their activated form as myofibroblasts) and macrophages elaborate excessive levels of matrix metalloproteinases (MMPs; eg, collagenase-1/matrix metalloproteinase-1, collagenase-3/MMP-13, gelatinase-A/MMP-2, and gelatinase-B/MMP-9) and cysteine endoproteases (cathepsins K and S), which corroborates the results of other studies.\textsuperscript{61,62–67} These
proteolytic enzymes degrade collagen and elastin in the extracellular matrix, which leads to vascular and valvular remodeling and subsequent structural changes. Activation of MMP-9 by osteopontin may play a role in aortic valve calcification by initiating elastin degradation that could be a nidus for hydroxyapatite crystal formation. Proteolytic activity affects not only the extracellular matrix but also other substrates (eg, interleukin [IL]-1β precursor, tissue factor pathway inhibitor), which may in turn enhance valve inflammation. Molecular imaging has thus enabled us to analyze proteolytic activity, which could provide diagnostic information on inflammation and matrix degradation and thereby predict risk of subclinical aortic valve stenosis.

**Imaging of Osteogenic Activity**

The need to closely follow and evaluate changes once lesions are identified in patients with aortic valve thickening that could eventually lead to aortic sclerosis is evident because of the high morbidity and mortality rates reported. The early stages of mineralization were observed with a bisphosphonate-conjugated imaging agent that binds to nanomolar concentrations of calcium hydroxyapatite complexes elaborated by valvular interstitial cells (eg, myofibroblasts). Like all bisphosphonates, this noncleavable pyrophosphate analog avidly binds calcium, thus accumulating at sites of active biominalization and osteogenesis as detected by alkaline phosphatase activity. This calcium imaging agent can be excited at NIRF wavelengths that are spectrally distinctive from other imaging agents (eg, macrophage-targeted or cathepsin-activatable agents), thus enabling simultaneous correlation of osteogenic activity with other biological processes. In our study, immunohistochemical analysis of osteogenic markers was used to validate data produced via biological processes. In our study, immunohistochemical analysis of osteogenic markers was used to validate data produced via biological processes. In our study, immunohistochemical analysis of osteogenic markers was used to validate data produced via biological processes. In our study, immunohistochemical analysis of osteogenic markers was used to validate data produced via biological processes.

**Inflammation-Dependent Mechanisms of Calcific Aortic Valve Disease**

Although the cause of mineral dysregulation in early aortic valve disease requires further investigation, molecular imaging has enabled simultaneous visualization of the roles of various cells and enzymes in the early stages of mineralization in vivo, which supports the concept of inflammation-dependent development of calcific aortic valve disease. In summary, atherogenic factors and mechanical forces may activate valvular endothelial cells and initiate recruitment of inflammatory monocytes/macrophages, which when activated produce a cocktail of pro-osteogenic cytokines, growth factors, and proteolytic enzymes. Extracellular matrix remodeling and thickening/stiffening of the leaflets due to proteolytic activity may result in valvular dysfunction and alterations of mechanical stresses across the valve leaflet. The resulting change in flow patterns may further induce inflammation and the activation of fibroblasts into myofibroblasts and subsequently into osteoblast-like cells through augmentation of the Runx2 pathway. The end result would be the deposition of calcium primarily in regions of high mechanical stress and eventual immobilization of the aortic leaflets due to increased stiffening (Figure 1).

**Monitoring Changes in Osteogenic Activity During Atherosclerotic Plaque Progression and After Antiinflammatory Treatment**

**Monitoring Calcification and Inflammation in Living Animals**

The limited knowledge regarding cardiovascular calcification has been blamed on the inability to spatially and temporally resolve and quantify the dynamic pro-osteogenic molecular mechanisms in vivo. These limitations can be overcome with the use of innovative molecular imaging tools to visualize and quantify components of inflammation and the osteogenic activity associated with early-stage atherosclerotic.
Although in vitro studies have suggested the potential role of inflammation in calcification,75–79 few in vivo reports pursued this idea. We therefore used molecular imaging to test the hypothesis in vivo that atherosclerotic plaque inflammation, determined as macrophage infiltration, triggers osteogenic activity and that further reduction of inflammation may decrease arterial calcification.34 Nanoparticle technology was once again used to image macrophages and the calcium imaging agent used to image the osteogenic differentiation of smooth muscle cells and areas of active mineralization processes in the arteries,23,34,42 which demonstrated the value of these imaging methods in analysis of calcific processes within both aortic valves and arteries. In this study, apoE/− mice were fed an atherogenic, high-cholesterol diet supplemented with atorvastatin.34 For the first time, intravitral microscopy was performed sequentially on the carotid arteries of untreated mice and a statin-treated cohort of mice at 20 and 30 weeks of age. Macrophage number was found to increase in association with advanced osteogenic signal by the later time period; however, this progression of macrophage burden and osteogenesis was prevented by antiinflammatory statin therapy, which further supported our hypothesis that inflammation may trigger calcification.

**Arterial Calcification as an Inflammatory Disease**

A series of groundbreaking in vitro studies by Demer’s group75–78 demonstrated that macrophage-derived cytokines (eg, IL-1β, IL-6, IL-8, tumor necrosis factor-α, insulin-like growth factor-1, and transforming growth factor-β) induce osteogenic differentiation and mineralization of vascular smooth muscle cells. The results of these studies produced the theory that proinflammatory cytokines promote atherosclerosis-associated calcification by regulating the differentiation of calcifying vascular smooth muscle cells. Our in vivo molecular imaging studies corroborated previous reports and further linked macrophages with osteogenesis.23,34 Fluorescence reflectance imaging ex vivo elegantly visualized the real-time association of inflammation and early calcification.34 Similar to macrophage and calcification signals noted in regions of high flexure and increased mechanical forces in the aortic valve, macrophage burden and osteogenic activity colocalized predominantly in proatherogenic regions of high mechanical stress, including the lesser curvature of the aortic arch, the aortic root, the innominate artery, the carotid bifurcation, and the aortic root (Figure 1). This evidence further supports the importance of macrophages in calcification.

The advancement of in vivo molecular imaging techniques has enabled us to further elaborate on the inflammation-dependent calcification paradigm (Figure 2), which can be split into 3 distinct phases: initiation, propagation, and end-stage calcification. We suggest that in the initiation phase, macrophage infiltration and inflammation precede calcification, and activated proinflammatory pathways induce osteogenic transformation of vascular wall cells. This phase can be characterized by the expression of pro-osteogenic cytokines (eg, IL-1β, IL-6, IL-8, tumor necrosis factor-α, insulin-like growth factor-1, and transforming growth factor-β) by macrophages and other inflammatory cells.5,30 In the propagation phase, vascular smooth muscle cells undergo osteogenic differenti-
microcalcifications, fibrous cap thinning, and eventually plaque rupture. Using molecular imaging at this stage, we showed that inflammation and microcalcification evolved within close proximity and overlapped at border regions and suggested that plaque ruptures may occur in these adjacent areas.34

The deposition of hydroxyapatite progresses quickly,34 and microcalcifications evoke additional proinflammatory responses from macrophages, which demonstrates that a positive-feedback loop of calcification and inflammation drives disease progression.87,88 Moreover, the onset of microcalcifications is an added complication, because they may cause plaque rupture as a result of debonding, which leads to acute clinical events as predicted by Vengrenyuk and colleagues.13,15,89 It is hypothesized, however, that reducing inflammation through antiinflammatory therapy at the early stages could retard subsequent osteogenesis and stabilize the plaque until further inflammatory events are initiated.54,42,50,84 Because reversing or halting the mineralization process in later stages of calcific disorders may be more difficult, the final of the 3 phases—end-stage calcification—is classically viewed as irreversible. This final phase is associated with advanced tissue mineralization and reduced inflammation and can be readily detected by conventional imaging approaches (eg, CT).90

Detecting Elastolysis-Triggered Calcification in Chronic Renal Disease
Cardiovascular disease is the most common cause of death in individuals with chronic renal disease (CRD).91–93 In addition to the classic risk factors mentioned previously, patients with CRD have hyperphosphatemia, an independent risk factor for cardiovascular death.94,95 Although cardiovascular disease in the general population is associated with older age, lesions of cardiovascular calcification have been reported in dialysis patients of a much younger age,95,96 which confirms that cardiovascular calcification is not simply a degenerative disorder. This issue, in our opinion, also heightens the necessity for research into this area. We have already highlighted the implication that proteolytic enzymes expressed by activated macrophages are involved in atherosclerotic plaque progression and aortic valve disease.62,97–100 Although the contribution of cathepsins to the pathogenesis of atherosclerosis and aortic stenosis has been established previously, our study using optical molecular imaging produced direct in vivo evidence for the role of cathepsin S,41 one of the most potent elastases, in accelerating arterial and valvular calcification in mice with atherosclerosis and CRD induced by 5/6 nephrectomy, a 2-step CRD-promoting procedure that is known to aggravate atherosclerosis and intimal calcification in apoE−/− mice.41,101

Role of Elastin Degradation in Calcification
Elastolytic cathepsin S is abundantly expressed by macrophages and smooth muscle cells in atheroma.102 Previous studies have suggested that the structure of the elastic fibers allows them to withstand proteolytic degradation,103,104 but during the progression of atherosclerosis, an imbalance in the regulation of cathepsins may enable proteases such as cathepsin S to degrade elastin.69,105 Elastolysis induced by inflammation in the atherosclerotic plaques may promote the release of biologically active, soluble, elastin-derived peptides that trigger the osteogenic differentiation of smooth muscle cells.41,106,107 These elastin-derived peptides act as biologically active molecules known as matrikines or elastokines, which can regulate cell processes such as migration and proliferation, as well as the release of bone-regulating proteins from myofibroblasts or smooth muscle cells, resulting in calcific lesions.106–110

Imaging Elastolytic Activity
Our study41 used a new protease-activatable imaging agent that was specific for cathepsin S.111 Molecular imaging detected increased cathepsin S and osteogenic activities in CRD mice, whereas calcification was decreased in atherosclerotic plaques and aortic valves in mice that lacked cathepsin S activity. These results were corroborated with optical projection tomography and quantitative histology and provided the first direct in vivo evidence for the role of cathepsin S in calcification.

We have previously shown that proteolytic activity, in the form of cathepsins (cathepsin S and cathepsin K) and MMPs (MMP-2 and MMP-9), plays a role in valvular diseases.62 Therefore, because mature aortic valves also have an elastin-rich structure100 and the ability to develop lesions similar to those of atherosclerotic plaques,23,34 we proposed that similar mechanisms of cathepsin S–associated elastin degradation contribute to the development of calcific aortic valve disease. In the early stages of aortic valve and artery calcification, macrophage-derived elastolytic enzymes participate in the degradation of elastin matrix. This remodeling of the matrix leads to proliferation of vascular smooth muscle cells or valvular myofibroblasts, which causes lesion formation and growth. The elastolysis-triggered release of biologically active peptides attracts more macrophages, which in turn produce more proteolytic enzymes, which promotes further expansion of the lesion. These biologically active peptides trigger osteogenic differentiation of the cells. Patients with CRD have the additional complication of hyperphosphatemia, a mineral imbalance that leads to osteolysis and increased calcium and phosphate levels in serum. These calcium and phosphate levels in turn accelerate calcification of vascular smooth muscle cells or valvular myofibroblasts.41,84,112

Inverse Correlation of Arterial and Aortic Valve Calcification With Osteoporotic Bone Remodeling: A Role for Inflammation
Clinical studies have suggested associations between cardiovascular calcification, atherosclerosis, CRD, and osteoporosis.113,114 Although this link initially was thought to be age related, epidemiological evidence has demonstrated an age-independent correlation between bone mineral density (BMD) and cardiovascular events.115–117 These studies noted a reduction in BMD along with arterial calcification in humans; this was corroborated by mouse studies that further demonstrated that atherosclerosis susceptibility corresponds with reduced bone mineralization.118,119 Limited studies suggest a mechanism behind this seemingly paradoxical event; a
recent literature review, for example, discussed the possibility that osteoporosis and cardiovascular calcification are tissue-specific responses to chronic inflammation. However, the precise nature of the reciprocal regulation of arterial calcification, calcific aortic valve disease, and bone osteogenesis remains unknown.

**Arterial, Valvular, and Bone Mineralization Have Shared Proinflammatory Mechanisms**

In our recent study, the relationship between cardiovascular calcification (arterial and valvular) and long bone remodeling (cortical and trabecular) was quantified simultaneously for the first time in mice with hyperlipidemia and with CRD by use of optical molecular imaging and high-resolution, 3-dimensional micro-CT. We hypothesized that cardiovascular calcification progresses with inflammation and correlates inversely with BMD. The study sought to provide mechanistic evidence on the role of inflammation in calcification and osteoporosis. Our results on the opposing effects of inflammation in cardiovascular organs (soft tissues) and in bone agree with previous reports. This study provided new insight into the relationship between osteoporosis and cardiovascular calcification and suggested shared inflammatory mechanisms of ectopic calcification and bone osteolysis. Direct comparison of macrophage burden and progression of osteogenic changes via NIRF imaging in each region of the same animals in vivo and ex vivo enabled us to discover that bone osteogenic activity and BMD decreased as atherosclerosis and calcific aortic valve disease developed and that the degree of cardiovascular calcification correlated directly with loss of BMD. Molecular imaging identified strong inflammatory activity in arteries, aortic valves, and long bones of mice with atherosclerosis and CRD, which demonstrates that inflammation at these 3 locations is related, probably via systemic or circulating inflammatory cues.

Osteoporosis was associated epidemiologically with atherosclerosis and hyperlipidemia many decades ago. More recently, a clinical study reported that stenotic coronary narrowing was more prevalent among women with low BMD, whereas preclinical studies have suggested that hyperlipidemia promotes cardiovascular calcification and reduces BMD via increased bone resorption. Other evidence suggests that osteoporosis may contribute to cardiovascular calcification by the release of biochemical factors, such as increased amounts of circulating phosphate and calcium and decreased amounts of parathyroid hormone, which promotes osteogenesis and mineralization of the arterial wall and aortic valve. This evidence agrees with results from studies showing that agents that block bone resorption in animals also block vascular calcification. Bisphosphonates, used in the management of osteoporosis, have been associated with decreased cardiovascular calcification in elderly subjects and increased prevalence of valvular, aortic, and coronary arterial calcification in younger women with subclinical cardiovascular disease. The reduction of cardiovascular calcification in elderly women may be due to decreased cholesterol levels and proinflammatory cytokines, or alternatively to declining bone resorption and subsequent decrease of circulating calcium phosphate. Additional studies are needed to elucidate the beneficial effect of bisphosphonates on bone metabolism in elderly patients; however, further evaluation of unfavorable effects in younger women is warranted.

The use of molecular imaging in our imaging study has strongly suggested that systemic and local inflammation, seemingly paradoxically, drive both cardiovascular calcification and bone loss. In simplified terms, inflammation causes differential tissue responses that result in “hardening” of soft tissue and “softening” of hard tissue, but it is unclear whether the pathways are similar. It has been suggested that inflammation in cardiovascular and bone regions may act through the nuclear factor-kB–RANK ligand pathway, but whether this mechanism is used simultaneously for cardiovascular calcification and osteoporosis is uncertain. Future studies could use molecular imaging to elucidate these signaling pathways, because there are still many questions to answer.

**Conclusions**

Both in vitro and clinical studies have suggested that a sequence of active osteogenic processes contributes to cardiovascular calcification and that osteogenic activity is initiated by inflammation. State-of-the-art multimodality molecular imaging has provided the opportunity to effectively visualize in vivo these different biological processes simultaneously using 2 spectrally distinct imaging agents and thus substantiate this theory. The studies cited in the present review have led us to the hypothesis that calcification of the artery and the aortic valve are mechanistically similar, and thus, the same sophisticated imaging modalities can examine both processes (Figure 3). A key finding of our studies was that molecular imaging techniques can visualize atherosclerotic plaques and aortic valve lesions in the early stages that are undetectable by conventional imaging modalities. However, the present review does not aim to lessen the value of studies that use histopathology and conventional imaging modalities but rather to support the development of innovative imaging techniques to enable further exploration of the pathogenesis of cardiovascular calcification.

Clinical trials have failed to demonstrate the benefit of statin therapy in slowing the progression of valve calcification. This may be due to the late implementation of the statins, after aortic valve calcification has progressed to the irreversible stage. Adjustment of atherogenic factors or the use of pharmacological therapies that target proinflammatory pathways may impede or even halt the progression of cardiovascular calcification when implemented during the early stages of calcification. For example, antiinflammatory therapies or the preservation of elastin integrity via the inhibition of elastolytic cathepsins such as cathepsin S might prevent cardiovascular calcification and its complications when introduced early. In addition, macrophage- or smooth muscle cell-targeted silencing of proinflammatory or pro-osteogenic factors with small interfering RNA may retard the progression of calcification. The combination of optical imaging agents with anticalcification drugs (eg, bisphosphonate) within the targeted construct may provide a unique platform for specific imaging and therapy for preclinical models and for future
clinical translation. Moreover, therapeutic interventions for calcification-prone patients with CRD could target inflammation, matrix degradation, or mineral imbalance. Further studies are required to establish the relationship between cardiovascular calcification and osteoporosis, to identify factors to target for the reciprocal regulation of these processes.

Molecular imaging, and particularly optical imaging, is anticipated to have the most impact on preclinical research, including identification of novel targets and mechanisms and evaluation of imaging tools in preclinical models. It is apparent that various limitations and issues need to be addressed before the molecular imaging approach can be used clinically. Despite this, several studies are producing favorable results in regard to the clinical translation of this evolving modality. Molecular imaging techniques that use radionuclides could be available for clinical use within the next 1 to 3 years, because many of the imaging probes have already been routinely used clinically. The complementary use of targeted molecular imaging agents with MRI has demonstrated promising results in animal studies. In addition, molecular imaging agents are being considered for clinical trials. Indeed, the use of cysteine protease–activatable NIRF agent is already believed to be clinically favorable; the backbone of this imaging agent has been found to be safe in clinical trials and the near-infrared fluorochromes are similar to those of an agent used widely in retinal angiography.

The development of promising, clinically feasible technology (eg, intravascular NIRF for coronary artery imaging55,56) is ongoing, and each study leads us closer to the clinical translation of this imaging modality.

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Disclosures
None.

References

Figure 3. Imaging of shared molecular mechanisms underlying arterial and aortic valve calcification. The studies cited in the present review showed that calcification is an active process composed of a sequence of events, initiated by inflammation and resulting in mineralization. Proinflammatory monocytes (1) are recruited to a site via activated endothelial cells (ECs). (2) The activation of ECs causes increased expression of adhesion molecules, such as vascular cell adhesion molecule-1, which can be visualized by NIRF imaging with a vascular cell adhesion molecule-1-targeted agent. Subsequent macrophage (3) accumulation follows, which can be analyzed with NIRF macrophage-targeted nanoparticles. The release of proteolytic enzymes, including matrix metalloproteases and cathepsins, by macrophages, which stimulates the differentiation of myofibroblasts (4) and smooth muscle cells (5) into osteoblasts, can be visualized by molecular imaging with activatable imaging agents. Osteogenic activity in the form of osteoblast (6) formation and microcalcifications associated with generation of calcified matrix vesicles (7) can be identified by a bisphosphonate-conjugated imaging agent, whereas apoptotic bodies (8) can be detected by a fluorescently tagged annexin A5 imaging probe. Macrocalcifications (9) can be readily detected by molecular imaging and conventional imaging techniques. This schematic visualizes the theory that calcification follows similar processes in both the artery and the aortic valve and summarizes how NIRF imaging can visualize dynamic sequences of calcification process. Illustration credit: Cosmocyte/Ikumi Kayama.


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