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Visualizing novel concepts of cardiovascular calcification

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Abstract

Cardiovascular calcification is currently viewed as an active disease process similar to embryonic bone formation. Cardiovascular calcification mainly affects the aortic valve and arteries and is associated with increased mortality risk. Aortic valve and arterial calcification share similar risk factors, including age, gender, diabetes, chronic renal disease, and smoking. However, the exact cellular and molecular mechanism of cardiovascular calcification is unknown. Late-stage cardiovascular calcification can be visualized with conventional imaging modalities such as echocardiography and computed tomography. However, these modalities are limited in their ability to detect the development of early calcification and the progression of calcification until advanced tissue mineralization is apparent. Due to the subsequent late diagnosis of cardiovascular calcification, treatment is usually comprised of invasive interventions such as surgery. The need to understand the process of calcification is therefore warranted and requires new imaging modalities which are able to visualize early cardiovascular calcification. This review focuses on the use of new imaging techniques to visualize novel concepts of cardiovascular calcification.

Introduction

Calcification, a progressive disease of dysregulated mineral metabolism, has been observed in the cardiovascular system for many decades. Ectopic calcification of the cardiovascular system predominantly affects the aorta, coronary arteries, peripheral arteries, and the aortic valve. Traditionally, cardiovascular calcification has been considered as a passive phenomenon associated with aging; however, it is currently viewed as an actively regulated disease process. More specifically, mounting evidence suggests that the underlying mechanisms of cardiovascular calcification are similar to embryonic bone formation (Demer and Tintut, 2008; Otto, 2008; Towler and Demer, 2011).

The pathological mineralization of the arteries is often observed in atherosclerotic plaques, which clinically translates to reduced compliance of the vessel wall, associated with hypertension (Abedin et al., 2004). In addition, studies demonstrate that microcalcification in the fibrous cap overlying the necrotic core of atherosclerotic plaques could lead to microfractures and plaque rupture, leading to acute thrombosis and possibly fatal myocardial infarctions (Vengrenyuk et al., 2006; Virmani et al., 2006). Furthermore, calcification poses significant challenges for the outcome of interventional strategies such as percutaneous coronary interventions for coronary artery disease (Moses et al., 2004). Calcification of the aortic valve, or calcific aortic valve disease (CAVD), progresses from mild thickening to severe calcification and leads to stiffening of the aortic valve leaflets, eventually causing left ventricular outflow obstruction and heart failure (Mohler, 2004; Rajamannan et al., 2007; Otto, 2008). Even mild aortic valve calcification is associated with increased mortality risk

(Lloyd-Jones et al., 2009). There are no known therapies that slow disease progression, and in case of aortic valve stenosis, surgical valve replacement and evolving transcatheter valve implantation (TAVI) are the only current treatments. Therefore, effective anti-calcification therapies are warranted.

Aortic valve calcification and arterial calcification share similar risk factors, such as age, gender, smoking, hypercholesterolemia, metabolic syndrome, end-stage renal disease, and diabetes mellitus (Stewart et al., 1997). Pathologically, explanted human stenotic aortic valves demonstrate similar lesions as observed in atherosclerotic plaques consisting of inflammatory cells and calcific deposits (Otto et al., 1994). Patients with familial hypercholesterolemia are prone to develop atherosclerosis in addition to developing valve lesions that calcify with age (Rajamannan et al., 2001a, 2001b). Moreover, preclinical animal studies show atherosclerotic-like lesion in aortic valve leaflets of rabbits and mice with established atherosclerosis (Rajamannan et al., 2002). Since aortic valve calcification and atherosclerosis potentially share a similar pathological mechanism, statins (3-hydroxy-3methylglutaryl-coenzyme A [HMG-CoA] reductase inhibitors) emerged as a therapeutic agent. Although several retrospective studies demonstrate a reduction in aortic valve stenosis when treated with statins (Aronow et al., 2001; Novaro et al., 2001; Bellamy et al., 2002), large prospective randomized clinical trials, do not support these findings and show no reduction in aortic valve calcification when treated with high doses of statins (Cowell et al., 2005; Rossebo et al., 2008). Although, this may be due to the late implementation of statins, after aortic valve calcification has progressed to an irreversible stage, these studies underscore our lack of understanding of underlying mechanisms of cardiovascular calcification. An increasing aging population necessitate further investigation of the pathways that contribute to cardiovascular calcification. The need to develop new therapeutic targets to prevent or reverse cardiovascular calcification warrants the use of novel imaging methods, as recently highlighted by the Working Group on Calcific Aortic Stenosis of the National heart, Lung, and Blood Institute (NHLBI) (Rajamannan et al., 2011).

Currently cardiovascular calcification is visualized by noninvasive conventional imaging modalities such as echocardiography, computed tomography (CT), and magnetic resonance imaging (MRI). Even though, CT and echocardiography are vital diagnostic tools because of their high sensitivity and ability to quantify calcium content of soft tissues, clinical imaging is usually only performed when patients are becoming symptomatic. It should be mentioned that coronary artery calcium (CAC) scoring is used in asymptomatic patients for risk stratification for CAD. Clinical symptoms arise only at advanced stages of calcification, leaving physicians too late to reduce or prevent calcification. In addition, CT and echocardiography have a relatively low spatial resolution and cannot detect early stages of cardiovascular calcification. Early detection methods are needed to not only elucidate early mechanisms of cardiovascular calcification, but also to establish whether the disease is reversible. Imaging modalities that can visualize molecular targets can contribute to understand important molecular and cellular aspects of cardiovascular calcification. The development of molecular imaging has enabled us to visualize early stages of calcification and piece together with the mechanism of disease progression leading to calcium deposition (Fig. 1). This review discusses how innovative imaging techniques have helped visualizing novel concepts of cardiovascular calcification.

Initiation of calcification

The mechanistic pathways involved in the development of cardiovascular calcification remain largely unknown. In calcification research much focus is shifting toward mapping the entire process of the disease and as such determining the initiation, progression and end-

stage of the disease process. The current pathological concept of cardiovascular calcification, albeit arterial or valvular in nature, is that cardiovascular risk factors lead to endothelial dysfunction, followed by the deposition of lipoprotein (LDL) particles and other compounds that trigger inflammatory response (Aikawa et al., 2007b; Otto, 2008; Towler, 2008; Rajamannan, 2010). The inflammatory state leads to the activation of inflammatory signaling pathways, macrophage infiltration and T-lymphocyte activation. Activation of inflammatory pathways contributes to the disease process, which in turn activates smooth muscle cells (SMC) in the arterial wall and valvular interstitial cells (VIC) in the aortic valve to differentiate to osteoblast-like cells and deposit calcium. Although the initiation of cardiovascular calcification is still a matter of investigation, evidence suggests that endothelial dysfunction leads to osteoblastic differentiation of underlying SMC and VIC in the arterial wall and aortic valve, respectively.

Endothelial activation

The endothelium maintains normal homeostasis at the vasculature–blood interface, however dysfunctional endothelial cells promote an inflammatory response and the onset of atherosclerosis/intimal calcification (Gryglewski et al., 1988). Noninvasive ultrasound molecular imaging, via targeted imaging agents, can detect lesion-prone vasculature before the appearance of advanced calcified atherosclerotic plaques (Kaufmann et al., 2010). Sites of atherosclerotic lesion formation are associated with an increased surface expression of endothelial cell adhesion molecules, such as VCAM-1 and P-selectin (Cybulsky and Gimbrone, 1991; Nahrendorf et al., 2006; Kaufmann et al., 2010). This expression of VCAM-1 by endothelial cells precedes fatty streak formation (Nakashima et al., 1998) and is up-regulated in response to oxidized LDL cholesterol – a hypercholesterolemic milieu (Marui et al., 1993). VCAM-1 participates in the initiation and progression of atherosclerotic plaques can be visualized in vivo by noninvasive molecular imaging (Fig. 2) (Cybulsky and Gimbrone, 1991; Nahrendorf et al., 2006; Kaufmann et al., 2010).

The mechanism of initiation of vascular calcification is proposed to be similar to that which we have suggested for calcific aortic valve disease (Fig. 3) (Aikawa et al., 2007a, 2007b; Aikawa and Otto, 2012) Activated endothelial cells recruit inflammatory cells, mainly in the form of monocytes/macrophages, which contribute to the progression of this pathological disorder. Our recent work used molecular imaging to detect early changes in aortic valve disease. Utilizing a similar approach as imaging arterial endothelial cells, VCAM-1 targeted agents are distributed in the commissures of the aortic valve (Nahrendorf et al., 2006), which was validated by immunohistochemical analysis of VCAM-1 expression (Aikawa et al., 2007b). It has been confirmed that valvular endothelial dysfunction or injury leads to increased expression of adhesion molecules VCAM-1, ICAM-1 and E-selectin (Ghaisas et al., 2000; Muller et al., 2000). Mechanically the commissures endure the greatest amount of stress during the cardiac cycle, making these areas more prone to endothelial injury than other parts of the leaflets. In addition, high pulsatile shear stress has demonstrated the upregulation of inflammatory receptors by valvular endothelial cells for circulating cytokines and inflammatory monocytes, leukocytes and T-lymphocytes (Muller et al., 2000; Shavelle et al., 2008; Sucusky et al., 2009).

Another endothelial mechanism suggested a role of reactive oxygen species (ROS) as a possible initiating factor of calcification (Sorescu et al., 2004; Butcher and Nerem, 2006; Sucusky et al., 2009). ROS, including oxidized lipids, can cause endothelial cell injury, leading to loss of cell alignment and upregulation of cell adhesion molecules for circulating inflammatory cells. To this end, a recent study demonstrated imaging ROS with allylhydrazine, a liquid compound that converts to nitrogen and propylene gas after reacting with ROS, in mice using a clinical echocardiography system. Allylhydrazine can be

encapsulated within liposomes and is able to detect micromolar concentrations of radical oxidants (Perng et al., 2012).

Progression of calcification – An inflammatory concept

Evidence suggests that arterial and valvular calcification follows a pathway similar to that of endochondral bone formation (Rattazzi et al., 2005); osteoblastic differentiation of vascular SMC is via the Runx2 pathway (Steitz et al., 2001) similar to VIC. A series of in vitro studies (Watson et al., 1994; Parhami et al., 2002; Tintut et al., 2002; Radcliff et al., 2005) demonstrate that monocytes and macrophages release inflammatory cytokines that promote cardiovascular calcification by regulating the differentiation of calcifying vascular cells. These calcifying vascular cells express a number of phenotypic markers synonymous with osteoblasts and chondrocytes (Aikawa et al., 2007a, 2007b; El-Abbadi and Giachelli, 2007; Peacock et al., 2010). Our molecular imaging studies on arterial intimal calcification have provided the first in vivo evidence of the role that inflammation plays in initiating calcification (Aikawa et al., 2007a). Nanoparticle technology can be used to image not only the accumulation of macrophages within the intima of the atherosclerotic plaque, but also the co-localization of macrophages with osteogenic activity or active mineralization. Intravital microscopy has been performed on the carotid arteries of untreated and statin-treated cohort of apoE^{-/-} mice at 20 and 30 weeks of age. Macrophage number, as visualized by macrophage-targeted iron-oxide nanoparticles, increased in association with osteogenic signal at 30 weeks. In this study of early calcification, anti-inflammatory statin therapy prevented both macrophage burden and osteogenic activity. Osteogenic differentiation of SMC and areas of active mineralization are effectively imaged via the use of a bisphosphonate-conjugated imaging agent (OsteoSense680) (Aikawa et al., 2007a; Hjortnaes et al., 2010a, 2010b). This imaging agent binds to calcium and will accumulate where there is increased osteogenesis, as confirmed by alkaline phosphatase activity (Kozloff et al., 2007).

The progression of arterial calcification has shown to largely depend on an inflammatory process, which in turn associates with proteolysis and tissue remodeling. Early calcifying atherosclerotic plaques, often termed “spotty” calcifications, are associated with microcalcification and numerous macrophages undergoing proteolysis (Ehara et al., 2004). These vulnerable early atherosclerotic plaques are at risk of fatal rupture (Vengrenyuk et al., 2006; Virmani et al., 2006; Demer and Tintut, 2008; Li et al., 2012). During the progression of atherosclerosis, macrophages elaborate proteolytic activity, in the form of elastases (e.g., cathepsin S) and metalloproteinases (e.g., MMP-1, MMP-2, MMP-9). Our study utilized a novel protease-activatable imaging agent specific for cathepsin S and provided the first direct in vivo evidence for the role of this elastase in cardiovascular calcification (Aikawa et al., 2009). Molecular imaging detected increased cathepsin S and osteogenic activities in CRD mice, induced by 5/6 nephrectomy, compared to control apoE^{-/-} mice. Furthermore, calcification was decreased in atherosclerotic plaques and aortic valves in mice lacking cathepsin S. These results were corroborated using optical projection tomography and quantitative histology.

In the early stages of cardiovascular calcification, macrophage-derived elastolytic and proteolytic enzymes degrade elastin and collagen, respectively, major components of the extracellular matrix (ECM) in both the aortic valve and arteries (New and Aikawa, 2011). Inflammation-induced elastolysis in the atherosclerotic plaques results in the release of biologically active, soluble elastin-derived peptides (Karnik et al., 2003; Simionescu et al., 2007). These biologically active peptides may regulate cell processes such as proliferation, as well as promote osteogenic differentiation of SMC and subsequent microcalcification formation (Saulnier et al., 1991; Karnik et al., 2003; Simionescu et al., 2007).

Microcalcifications provoke a pro-inflammatory response in macrophages via mechanisms involving protein kinase C- α and ERK1/2 MAP kinase (Nadra et al., 2005, 2008). Therefore a positive feedback loop of inflammation and calcification drives progression of arterial calcification (Bostrom, 2005; Nadra et al., 2008), corroborated in vivo via the use of molecular imaging in our study (Aikawa et al., 2007a). Molecular imaging is capable of characterizing/diagnosing plaque activity, in the form of proteolytic activity, to identify high-risk patients. Thus the visualization of proteolytic activity, via the use of near-infrared fluorescence (NIRF) beacons/probes, may prove a valuable tool in the diagnosis of vulnerable rupture-prone plaques and onset of aortic stenosis.

The inflammatory cascade, that is initiated by the valvular endothelium, first results in excessive remodeling of the aortic valve extracellular matrix, which follows the clinical observation of aortic fibrosis preceding aortic valve stenosis (Otto et al., 1994). Similar to the arterial wall, proteolytic enzymes and their inhibitors maintain a balance that can degrade ECM compounds such as collagen and elastin during valvular remodeling. Using macrophage-targeted NIRF-conjugated iron nanoparticles and protease-activatable NIRF probes, we have demonstrated that macrophages correlate with increased levels of matrix metalloproteinases (MMP-1, MMP-2, MMP-9, MMP13,) and cysteine proteases (cathepsin S, cathepsin K) in early stage calcification animal models (Aikawa et al., 2007a, 2007b, 2009). In turn, we have also showed that inflammatory activity correlated with osteogenic activity using the same previously described calcification sensitive imaging probe (Hjortnaes et al., 2010). This imaging agent uses different NIRF wavelengths than other imaging agents, allowing for the correlation of osteogenic activity with other pathological processes of the valve. Using this approach, we have correlated inflammation with early osteogenic activity in the valve, and confirmed by alkaline phosphatase specific activity and immunohistochemistry for osteocalcin, osteopontin, and the osteogenic transcription factor Runx2 (Aikawa et al., 2007b).

Furthermore, it is also possible to use these NIRF agents for MRI visualization, making the translation to clinical practice very feasible. Recently developed iron-oxide based magnetic nanoparticles, which are engulfed by tissue macrophages by phagocytosis, can be used for T2 weighted contrast MRI and PET (Jaffer et al., 2006). This technology allows clinicians to discover a potential reversibility of the inflammatory response that leads to cardiovascular calcification by visualizing molecular, anatomic and physiological changes of the cardiovascular system. Another optical imaging technology, microoptical coherence tomography (micro-CT), has recently been developed and is able to visualize microcalcifications, cholesterol crystals, macrophages and proteins with a 1- μ m resolution. It does not require the injection of molecular imaging agents and can thus be more easily translated from bench to bed. Nevertheless both NIRF imaging and micro-OCT have limited tissue penetration.

Recent evidence suggests that positron emission tomography (PET) imaging could overcome this limitation by using PET tracers that target inflammation and calcification (Beheshti et al., 2011; Folco et al., 2011; Aikawa and Otto, 2012; Hyafil et al., 2012). Glucose analogue 18F-fluorodeoxyglucose (18F-FDG) is an imaging agent taken up by cells by glucose transport proteins, upon which the agent becomes trapped in macrophages. 18F-sodium fluoride (18F-NaF) binds to hydroxyapatite and is a marker for mineralization. A recent study that used this imaging approach confirmed, in patients with the full spectrum of aortic valve calcification, that inflammation and mineralization are present in early stages of aortic valve calcification (Dweck et al., 2012). It is the first clinical study to visualize early calcification processes in patients that confirms our current understanding of aortic valve calcification.

Although the exact role of inflammation remains to be elucidated, it is clear that specific imaging agents can be used to visualize mechanisms related to cardiovascular calcification. Moreover, specific targeting imaging agents, for particular molecules of interest, may prove beneficial as a means to visualize potential biomarkers or therapeutic targets.

Alternative mechanisms of arterial calcification

Although we are fully in support of the widely accepted concept that calcifying vascular cells play an important role in cardiovascular calcification, we also fervently believe that there are alternative mechanisms of mineralization. The apoptosis of macrophages has been proposed to contribute to plaque vulnerability (Virmani et al., 2006). In addition, macrophages induce the apoptosis of vascular SMC by direct interaction (Boyle et al., 2001, 2002). The release of apoptotic bodies from macrophages and SMC undergoing apoptosis may provide a nidus for calcification and provoke the generation of microcalcification (Proudfoot et al., 2000; Shanahan, 2007). Apoptosis can be monitored in the vasculature in realtime via the use of molecular imaging approaches with fluorescently tagged annexin V imaging agents (van Tilborg et al., 2010; Corsten and Bennaghmouch, 2011). The clinical translation of imaging approaches such as these may prove beneficial in assessing the effectiveness of specific therapies, as well as a means to visualize disease progression.

Osteoblasts and chondrocytes release hydroxyapatite-nucleating matrix vesicles during biological skeletal tissue mineralization (Anderson, 1981; Anderson et al., 1990). Matrix vesicles (30–300 nm), precursors of microcalcifications, bud from the plasma membrane of living cells and also believed to serve as a nidus for mineral nucleation in ectopic calcification (Kim, 1976; Anderson, 2003; Bobryshev et al., 2007). Evidence suggests that these matrix vesicles contain inhibitors of mineralization, such as Matrix Gla Protein and Fetuin-A, and a reduction in these inhibitors enhances their calcific potential (Reynolds et al., 2004; Chen et al., 2008; Kapustin et al., 2011). Calcifying matrix vesicles have been stated to possess exposed phosphatidylserine (Kapustin et al., 2011), therefore some of the current molecular imaging modalities used to image apoptosis and calcification can also detect matrix vesicle accumulation. The exact role that these vesicles play in cardiovascular calcification and the mechanism by which they are released still require further investigation.

Late-stage calcification

Late-stage cardiovascular calcification is characterized by advanced tissue mineralization and diminished inflammation. It is classically reviewed as irreversible due to the development of advanced calcified material and is readily detected by conventional imaging approaches (e.g., echocardiography, CT) (Johnson et al., 2006). All in all, cardiovascular calcification, albeit arterial or valvular, seems to follow similar pathways towards mineralization (Fig. 3). Specifically, calcification starts with endothelial dysfunction or activation due to inflammation, leading to an increased uptake of inflammatory cells. Inflammation therefore can be viewed as a propagation process eventually leading to the differentiation of SMC or VIC to actively deposit calcium in the arterial wall and aortic valve, respectively. Where conventional imaging modalities are limited, this early calcification process can be visualized by molecular imaging. Table 1 depicts molecular agents used in detecting various components of cardiovascular inflammation and calcification.

Conclusion

Cardiovascular calcification is currently viewed as an active disease process. Understanding the pathological pathways from initiation to late-stage mineralization is key to identifying

therapeutic targets that can potentially treat cardiovascular calcification. Especially mapping early calcification before the disease process has passed a point of no return provides the window for potential treatment. Therapeutic agents have thus far not proved beneficial in the clinical setting, mainly because of late clinical diagnosis. In addition, because histopathological examination is not possible during longitudinal noninvasive imaging studies (e.g., echocardiography, CT, PET), validated high-resolution invasive imaging modalities (e.g., molecular imaging, micro-OCT) may be required to understand the mechanisms at various stages of CAVD.

The molecular imaging approach can unmask/resolve early pathophysiological changes in the cardiovascular system and may prove beneficial diagnostically in a clinical setting. Moreover, considering the amount of evidence demonstrating the role of inflammation in early stages of calcification (e.g., initiation, propagation), targeting inflammation could be beneficial in reducing and even halting subsequent mineralization. Although current clinical imaging approaches, such as echocardiography, remain essential for clinical management and for following CAVD progression once valve obstruction is present, PET may be the method of choice for detection of early calcification and inflammation in clinical trials. Molecular imaging modalities possess great potential in visualizing *in vivo* cardiovascular calcification and will be vital in the development of therapeutic targets. Large, prospective, event-driven studies of noninvasive imaging techniques will be required to determine the place of these modalities in future clinical practice.

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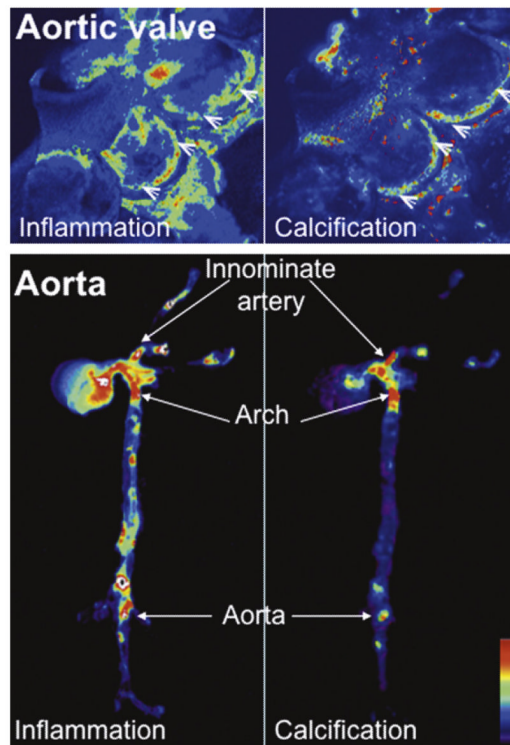


Fig. 1. Molecular imaging of cardiovascular calcification. Molecular imaging visualizes inflammatory activity, defined as macrophage accumulation, and osteogenesis in the aortic valves and aortas of apoE^{-/-} mice. Magnetofluorescent nanoparticles were injected into mice to visualize macrophage accumulation (left). Spectrally distinct bisphosphonate-imaging agent that binds to hydroxyapatite were injected to detect osteogenic activity (right). Inflammatory and osteogenic activity colocalized in the aortic valve (top) and in the aorta (bottom), specifically in the areas of highest mechanical stresses at the aortic valve attachment (arrowheads) and at the atherosclerosis-prone areas, such as the innominate artery, aortic arch, and abdominal aorta (arrows). High signal intensities are shown in red/yellow/green (New and Aikawa, 2011).

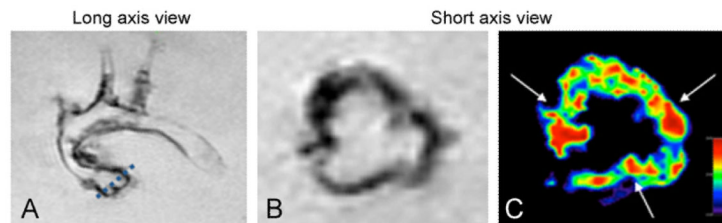


Fig. 2. Visualization of VCAM-1 targeted agents. MRI visualizes endothelial activation detected by VCAM-1-targeted agent, in the commissures of aortic valves, which are regions of high flexure and mechanical stress: (A) dotted line = slice position of short axis view, (B) negative signal enhancement caused by uptake of VCAM-1-targeted nanoparticles, and (C) color-coded signal (red) show focused uptake of VCAM-1 in commissures (arrows) (Aikawa et al., 2007b).

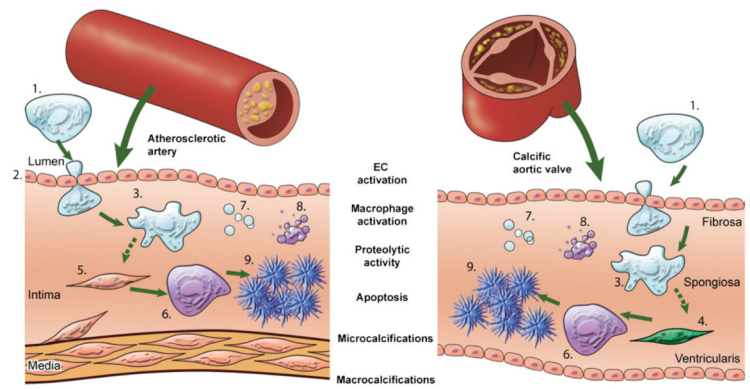


Fig. 3. Mechanisms underlying arterial and aortic valve calcification. This figure depicts the theory that cardiovascular calcification follows similar pathways in both the artery and the aortic valve and summarizes how molecular imaging can visualize concepts of the calcification process. Cardiovascular calcification is an active process, initiated by endothelial dysfunction/activation and/or by inflammation and resulting in mineralization. Pro-inflammatory monocytes (1) are recruited to a site via activated/injured endothelial cells (ECs). (2) The activation of ECs causes increased expression of adhesion molecules, such as VCAM-1, which can be visualized by NIRF imaging VCAM-1 agent. Subsequent macrophage (3) accumulation follows, which can be analyzed with NIRF macrophage-targeted nanoparticles (AminoSPARK). 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 (MMPSense, ProSense). 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 (OsteoSense) whereas apoptosis (8) can be detected by a fluorescently tagged Annexin A5 imaging probe. Calcification (9) can be readily detected by molecular imaging and conventional imaging techniques such as CT and Echocardiography (New and Aikawa, 2011).

Table 1

Molecular imaging agents in cardiovascular calcification

Cardiovascular calcification	Target	Imaging agent	Imaging modality
Processes associated with early calcification			
Endothelial dysfunction	VCAM-1	VINP-28 Microbubbles	Optical imaging ^a /MRI US
	ICAM-1	NIRF Microbubbles	Optical imaging US/MRI
	ROS	Microbubbles MPO ^b probe oxLDL-targeted nanoparticle	US Optical imaging MRI
Inflammation	Macrophages	Nanoparticles (e.g., CLIO ^b)	Optical Imaging/MRI/PET
	MMP	MMPsense	Optical imaging
	Cathepsin	ProSense	Optical imaging
Progression of calcification			
Inflammation	Macrophages	Nanoparticles (e.g., CLIO ^b)	Optical imaging/MRI/PET
Apoptosis	Annexin V	Nanoparticle	Optical imaging/MRI
Microcalcification	Caspase	18F-FDG	PET
	Hydroxyapatite	Activatable probes OsteoSense 18F-NaF	Optical imaging/PET Optical imaging PET
Late-stage calcification			
Advanced calcification	Hydroxyapatite	OsteoSense 18F-NaF	Optical imaging PET
	Calcium	–	Echocardiography CT

^aOptical imaging indicates one or more of the following modalities: Optical projection tomography (OPT), micro-optical coherence tomography (micro-OCT), fluorescence molecular tomography (FMT) and intravital microscopy (IVM).

^bNIRF, near-infrared fluorescence; MRI, magnetic resonance imaging; US, ultrasound; ROS, reactive oxygen species; MPO, myeloperoxidase; CLIO, cross-linked iron oxide; PET, positron emission tomography; FDG, fluorodeoxyglucose; NaF, sodium fluoride; CT, computed tomography.