Plaque Calcification During Atherosclerosis Progression and Regression

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Plaque calcification develops by the inflammation-dependent mechanisms involved in progression and regression of atherosclerosis. Macrophages can undergo two distinct polarization states, that is, pro-inflammatory M1 phenotype in progression and anti-inflammatory M2 phenotype in regression. In plaque progression, predominant M1 macrophages promote the initial calcium deposition within the necrotic core of the lesions, called as microcalcification, through not only vesicle-mediated mineralization as the result of apoptosis of macrophages and vascular smooth muscle cells (VSMCs), but also VSMC differentiation into early phase osteoblasts. On the other hand, in plaque regression M2 macrophages are engaged in the healing response to plaque inflammation. In association with the resolution of chronic inflammation, M2 macrophages may facilitate macroscopic calcium deposition, called as macrocalcification, through induction of osteoblastic differentiation and maturation of VSMCs. Oncostatin M, which has been shown to promote osteoblast differentiation in bone, may play a pivotal role in the development of plaque calcification. Clinically, two types of plaque calcification have distinct implications. Macrocalcification leads to plaque stability, while microcalcification is more likely to be associated with plaque rupture. Statin therapy, which reduces cardiovascular mortality, has been shown to exert its dual actions on plaque morphology, that is, regression of atheroma and increment of macroscopic calcium deposits. Statins may facilitate the healing process against plaque inflammation by enhancing M2 polarization of macrophages. Vascular calcification has pleiotropic properties as pro-inflammatory “microcalcification” and anti-inflammatory “macrocalcification”. The molecular mechanisms of this process in relation with plaque progression as well as plaque regression should be intensively elucidated.

Key words: Inflammation, Macrophage polarization, Microcalcification, Macrocalcification

Introduction: Historical Changes in the Concept of Vascular Calcification

Vascular calcification is a hallmark of atherosclerosis and is associated with adverse cardiovascular events. Particularly, in patients with diabetes mellitus and chronic kidney disease (CKD) vascular calcification is remarkably accelerated and contributes to a higher risk of cardiovascular morbidity and mortality¹, ². In coronary arteries, calcification is mainly localized within intimal plaque lesions except in CKD patients who develop medial as well as intimal calcification³.

In the eighteenth century, calcified lesions in the coronary arteries were described as the primary pathological features of coronary atherosclerosis⁴. In these early reports based on gross inspection of the coronary arteries, calcification was considered to be “the very essence of coronary sclerosis”⁴. Moreover, the fact that complete bone tissue forms within atherosclerotic artery wall has been already known since at least the 1800s⁵. Benivieni and Fallopio in the 1500s were the first to document a description of atherosclerosis as a degeneration of “arteries into bone,” which was called at that time ossification of the arteries. In 1863, Virchow stated that the vascular changes were ossification, not
Plaque Calcification and Atherosclerosis

Calcification, since they occurred by the same mechanism by which an osteophyte forms calcium on the surface of bone, and, in 1906, Bunting demonstrated the presence of bone marrow within a sclerotic aorta. Therefore, in those times vascular calcification was recognized as an osteogenic process that is an essential part of coronary atherosclerosis.

However, for most of the 20th century, vascular calcification has been considered to be a passive, unregulated, degenerative process occurring at an end stage of atherosclerotic plaque formation, because the paradigm for pathogenesis of atherosclerosis shifted towards "lipid hypothesis" that high blood cholesterol levels contribute causally to atherosclerosis. Late in the last century, Demer and colleagues re-evaluated the role of ossification in atherosclerotic calcification and put forward the concept that vascular calcification recapitulates osteogenesis. Over the last two decades, vascular calcification has been increasingly recognized as an active, organized, regulated, and preventable process with remarkable similarities to bone formation. On the other hand, since it was clarified that inflammation plays a major role in all phases of atherosclerosis, the inflammatory factors derived from macrophages to induce atherosclerotic plaque calcification have been extensively explored. Macrophage-derived cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and oncostatin M (OSM) have been identified to promote osteogenic differentiation and mineralization of vascular smooth muscle cells (VSMCs). The recent novel molecular imaging studies of atherosclerotic plaques in humans and experimental animals revealed the relationship between plaque inflammation and calcification. The results suggest that atherosclerotic plaque inflammation may precede active calcification. Therefore, the inflammation-dependent vascular calcification paradigm has been suggested.

Plaque Calcification in Association with Progression and Regression of Atherosclerotic Lesions

1. Macrophage Polarization and Plasticity in Atherosclerosis

Macrophages play a key role in inflammation, host defense, and tissue homeostasis. As a consequence, macrophages have a high plasticity and are able to adapt their phenotype, as instructed by their microenvironment and in agreement with their functional requirements. Conventionally, macrophages have been classified in two main groups, representing the extremes of a continuum, namely "classically activated" or M1 and "alternatively activated" or M2 macrophages. Upon stimulation with interferon-γ and toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS), macrophages obtain a pronounced pro-inflammatory M1 phenotype, characterized by the secretion of pro-inflammatory cytokines and reactive nitrogen and oxygen species. Conversely, the Th2 cytokines such as IL-4 and IL-13 polarize macrophages toward an anti-inflammatory M2 phenotype. M2 macrophages are involved in tissue remodeling and exhibit phagocytic activity. Although this dichotomous M1-M2 model is an oversimplification that only represents two extremes in a spectrum of macrophage activation states, it has been found that under pathological conditions, macrophages in vivo regularly mimics these two polarization states.

Macrophage polarization and plasticity in atherosclerosis have also been documented. Histological analysis of mouse and human plaque lesions has shown the presence of both M1 and M2 macrophages. In human plaques, M1 macrophages are the predominant phenotype in rupture-prone shoulder regions, whereas M2 markers are predominant in the adventitia and in stable cell-rich areas of the plaque. In another study of human plaques, M2 macrophages were found located far from the lipid core of the plaque. In mouse plaques, macrophages have M2 phenotypes at the early stages of the disease but M1 macrophages become dominant as the lesions advance. In a mouse model of plaque progression, M1 macrophages are enriched in vulnerable plaques induced with high-cholesterol diet and perivascular carotid collar placement. Furthermore, in a mouse model of regressing atherosclerotic plaques, there is reduced expression of maker genes characteristic of M1 macrophages, such as macrophage chemoattractant protein-1 (MCP-1), TNF-α, and inducible nitric oxide synthase (iNOS), coincident with increased expression of genes encoding markers of M2 macrophages, such as arginase-1, mannose receptor, and IL-10. These data suggest that M1 macrophages predominate in progression and contribute to the inflammatory state, whereas M2 macrophages are enriched in many models of regression and appear to participate in inflammation resolution and plaque remodeling.

2. Roles of Endoplasmic Reticulum (ER) Stress and Macrophage Apoptosis in Progression of Plaque Lesions

Atherosclerosis is a chronic inflammatory disease in which macrophages play a major role in its developmental processes. Macrophages are actively engaged in all stages of atherosclerosis development from plaque initiation to the transition to vulnerable plaque. Plaque inflammation characterized by macrophage infiltration can promote necrotic core formation and, in turn,
Microcalcification: Plaque Calcification Induced protein 27). In an IRE1 (inositol-requiring 1), and ATF6 (activating transcription factor 6). Activation of PERK leads to phosphorylation of the 80S ribosome and protein synthesis. Phosphorylation of the eIF2α-subunit of the eukaryotic initiation factor 2 (eIF2α), which inhibits its assembly of the 80S ribosome and protein synthesis. Phosphorylated eIF2α specifically promotes the translation of activating transcription factor 4 (ATF4). ATF4 is a pivotal transcription factor which plays an important role in osteoblast differentiation and bone formation. During osteoblast differentiation, ER stress occurs and activates the PERK-eIF2α-ATF4 signaling pathway followed by the promotion of gene expression essential for osteogenesis, such as osteocalcin and bone sialoprotein 27. In an in vitro model of vascular calcification, activation of PERK-eIF2α-ATF4 signaling also contributes to TNF-α-induced osteoblastic differentiation of VSMCs 28. Therefore, ER stress may induce plaque calcification through promoting osteoblastic differentiation of VSMCs.

(4) Microcalcification: Plaque Calcification Induced by Atherosclerotic Inflammation

In the progression of plaque lesions with necrotic core, the early phase of calcification, called as microcalcification, is initiated within apoptotic bodies and matrix vesicles released during the death of macrophages and VSMCs and these vesicular structures serve as nucleating sites for calcium phosphate crystal formation. Histologically, early calcification is observed within lipid pools as stippling by calcium stains which likely originates from SMC apoptosis or matrix vesicles and is generally $\geq 1 \mu m$ in size that can be appreciated by light microscopy 29. This microcalcification is not detected using standard non-invasive imaging techniques such as computed tomography (CT) and is thought to be associated with plaque vulnerability and an increased risk of rupture. Furthermore, microcalcification itself may provoke a pro-inflammatory response in macrophages and exacerbate plaque inflammation, leading to plaque rupture 30.

These microcalcifications often coalesce into larger masses and involve both necrotic core and the surrounding collagen-rich extracellular matrix to form speckled and fragmented of calcification 29. The progression of calcified lesions may proceed through osteogenic transdifferentiation of VSMCs by the action of pro-inflammatory cytokines such as TNF-α and OSM produced from macrophages with M1 phenotype. These cytokines induce expression of Runx2 and alkaline phosphatase and promote further mineralization within plaque lesions. However, chronic inflammatory conditions sustained within progressive plaque lesions may impair ordered osteogenesis as well as osteoblastic maturation of VSMCs, resulting in disorganized and fragmented calcified deposits, since unregulated and persistent chronic inflammation has been demonstrated to delay healing responses to bone injuries 31, 32.

(5) Macrocalcification: Plaque Calcification Associated with a Healing Response to Atherosclerotic Inflammation

From the teleological point of view, calcification is generally regarded as an important component of the healing process, generated as a physiologic defense designed to encompass, wall off and stabilize injured or damaged tissue and/or foreign agents 33. This is best exemplified by tuberculosis where the body attempts to wall off the intense necrotic inflammation associated with caseating granuloma using calcification. Similar mechanisms are believed to occur as a healing response to intense inflammation within the necrotic core in atherosclerosis 34.

In many tissues, the healing process progresses by overlapping phases of acute inflammation, resolving inflammation, proliferation, and remodeling. During these different phases of repair, M1 macrophages are essential to initiate the inflammatory response after injury, while switching to an M2 phenotype is fundamental for the resolution of inflammation and the regeneration of injured tissues. In the healing response of inflamed and/or injured (rupture) plaque lesions, the phenotypic switching of macrophages to M2 polar-
ization induces the resolution of inflammation and regression and/or stabilization of the plaque. A fundamental function of M2 macrophages in resolution is phagocytosis of necrotic cellular debris and apoptotic cells within the lesions. IL-10 secreted by M2 macrophages plays a major role in promoting to resolve plaque inflammation. In the later phases of the healing process, M2 macrophages not only support to produce extracellular matrix, but also facilitate plaque calcification through promoting osteoblastic differentiation and maturation of VSMCs. This type of macroscopic calcification, called as macrocalcification, is readily identified using standard x-ray angiography and CT and is widely believed to stabilize the plaque.

(6) Differential Contribution of M1/M2 Macrophages to Osteogenic Differentiation of VSMC

In the process of bone fracture healing, inflammation is thought to be an essential process that precedes bone formation and remodeling. During this process, macrophages are one of the earliest cell types that emerge in the injured tissue and are present throughout the healing process. M1 macrophages preferentially infiltrate into the bone fracture site in the acute phase, whereas M2 macrophages increase in number in the subacute phase. Since temporal depletion of macrophages in either the acute or subacute phase leads to significant delay of bone healing, it is suggested that both M1 and M2 macrophages play significant roles in the healing process by regulating the early and later phases, respectively. A number of soluble factors derived from macrophages, including OSM, have been shown to promote osteoblast differentiation of mesenchymal stem cells (MSCs). Recent studies have demonstrated the effects of different macrophage subtypes on osteogenic differentiation of MSCs or MC3T3 preosteoblasts in co-culture models. M2 macrophages promoted the proliferation and osteogenic differentiation of MSCs, while M0 (phorbol ester-treated THP-1 cells) and M1 macrophages solely stimulated the osteogenic differentiation of MSCs in the early and middle stages during co-culture. Similarly, osteogenesis by MC3T3 cells co-cultured with M1 macrophages was further enhanced by the subsequent conversion to M2 phenotype by the treatment with IL-4 at 72 hours after seeding. These data suggest that macrophages play a critical role in the osteogenic differentiation of progenitor cells and that the biological transition from a transient inflammatory (M1 polarization) to a tissue regenerative (M2 polarization) microenvironment at bone healing site is necessary for optimal bone regeneration.

In the inflammatory plaques with M1 macrophage predominance, pro-inflammatory cytokines such as TNF-α, IL-1β, and OSM and ER stress may promote only early phases of osteogenic differentiation of VSMCs as well as vesicle-mediated calcification as the result of apoptosis of macrophages and VSMCs (Fig. 1). These micro-calcified deposits formed under persistent pro-inflammatory conditions do not form any organized architectures to stabilize the plaque lesions as mentioned earlier. On the other hand, once phenotypic switch of M1 macrophage to M2 polarization occurs as the result of resolution of plaque inflammation, plaque healing and remodeling responses may proceed in parallel with osteoblastic differentiation of VSMCs and matrix mineralization. In this case, macroscopic calcification associated with organized structures reminiscent of authentic bone tissue may be elaborated by osteoblast-like cells, consequently contributing to plaque regression and stabilization (Fig. 1).

(7) Roles of OSM in Plaque Biology and Calcification

OSM is an inflammatory cytokine that belongs to the IL-6 class of cytokines. It is mainly produced by macrophages, neutrophils, and T lymphocytes and exerts many unique biological activities in inflammation, remodeling of extracellular matrix and modulation of cell growth and differentiation. OSM is expressed in human and mouse atherosclerotic lesions and may contribute to the progression of atherosclerosis through the STAT pathway. OSM facilitates tissue repair and remodeling responses after tissue injuries such as skin wound, myocardial infarction, and bone fracture. Moreover, thrombin up-regulates OSM expression in both human monocyte-derived and plaque macrophages. Therefore, OSM secreted by macrophages may promote healing response within the plaque subsequent to atherothrombosis due to plaque injury or rupture.

Recent evidences suggest that OSM may induce M2 polarization of macrophage phenotype. OSM stimulates the expression of M2 markers such as IL-10, arginase-1, and CD206 in RAW264.7 and peritoneal exudate macrophages in vitro and also polarizes the phenotypes of adipose tissue macrophages to M2 in C57BL/6J mice. Additionally, OSM secreted by hypoxia-primed cancer cells polarize macrophages to pro-angiogenic M2 subtype. In a co-culture model, we have identified OSM as one of the soluble factors derived from macrophages to induce calcifying phenotype in human VSMCs and have further clarified that OSM directly promotes osteoblastic differentiation of human VSMCs through JAK3-STAT3 pathway. Therefore, it is suggested that OSM may play a pivotal role in the development of plaque calcification associated with atherosclerosis progression and regres-
By contrast, microcalcification represents the early stage of plaque calcification that is initiated within apoptotic bodies and matrix vesicles released during the death of macrophages and VSMCs. This type of calcification, also called as spotty calcification, is associated with high risk and culprit atherosclerotic plaque in histopathologic and imaging studies using intravascular ultrasound (IVUS) and optical coherence tomography (OCT). Although the precise mechanism by which microcalcification contributes to plaque vulnerability remains to be clarified, recent studies have suggested that the presence of microcalcification in the fibrous cap of atheroma may intensify mechanical stresses on the surface of the fibrous plaque, leading to plaque rupture.

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**Plaque Calcification and Clinical Implications**

(1) Clinical Implications of Coronary Artery Calcification

Macroscopic calcification in coronary artery plaque lesion is easily detected with coronary CT and is widely believed to stabilize the plaque. Coronary artery calcium (CAC) measured by CT is closely related to atherosclerotic plaque burden and has strong predictive value for incident cardiovascular disease (CVD) events. However, emerging evidences suggest that greater calcium density in plaques is associated with decreased CVD risk. Of note, extensively calcified plaques themselves only rarely result in rupture and adverse events, but rather calcium scoring acts as a biomarker of overall disease and coronary plaque burden.

By contrast, microcalcification represents the early stage of plaque calcification that is initiated within apoptotic bodies and matrix vesicles released during the death of macrophages and VSMCs. This type of calcification, also called as spotty calcification, is associated with high risk and culprit atherosclerotic plaque in histopathologic and imaging studies using intravascular ultrasound (IVUS) and optical coherence tomography (OCT). Although the precise mechanism by which microcalcification contributes to plaque vulnerability remains to be clarified, recent studies have suggested that the presence of microcalcification in the fibrous cap of atheroma may intensify mechanical stresses on the surface of the fibrous plaque, leading to plaque rupture.

(2) Positron Emission Tomography (PET)/CT Imaging of Plaque Microcalcification Using $^{18}$F-Sodium Fluoride ($^{18}$F-NaF)

Positron emission tomography (PET)/CT imaging of atherosclerosis using $^{18}$F-sodium fluoride ($^{18}$F-
NaF) has recently been reported to have the potential to non-invasively identify high-risk microcalcification. 18F-NaF is a positron-emitting bone-seeking agent utilized clinically to identify primary bone tumors and skeletal metastases and its uptake reflects blood flow and osteoblastic activity. 18F-NaF uptake involves the exchange of fluoride ions with hydroxy groups in hydroxyapatite. A recent clinical study has shown that NaF uptake localizes to recent plaque rupture in patients with acute myocardial infarction and in those with symptomatic carotid disease. However, the precise mechanisms underlying 18F-NaF uptake in the vasculature remain to be elucidated. Irkle et al. demonstrated that 18F-NaF adsorbs to calcific deposits within plaque with high affinity and is selective and specific. Moreover, 18F-NaF PET/CT imaging was able to distinguish between areas of macro- and microcalcification. Preferential adsorbing of 18F-NaF to microcalcification is explained by its greater surface area and no barriers to penetration of the tissues. In contrast, there is the relatively small surface area accessible to 18F-NaF and much of the hydroxyapatite is internalized and not available for binding in macrocalcifications, so that 18F-NaF uptake is only observed at the periphery. Therefore, 18F-fluoride is the only currently available clinical imaging platform that can non-invasively detect microcalcification in active unstable plaque lesion.

(3) Optical Coherence Tomography and Plaque Calcification

Recently, OCT has emerged as the premier intracoronary imaging technology with a higher resolution (10–20 µm) than IVUS (100–200 µm) and also when compared with noninvasive CT and magnetic resonance imaging, both with a lower resolution (1 mm). It cannot detect a cell apoptosis based microcalcification because of its size (<10–20 µm). To the contrary, OCT can show spotty or speckled calcification which is smaller than macrocalcification but larger than microcalcification. Definition of spotty or speckled calcification has not yet been established, but some studies defined spotty calcification as <4 mm or <2 mm. This size calcification related with total burden of calcium.

Zeng et al. reported that serial OCT analysis assessed calcium growth with fused grayscale IVUS, IVUS–virtual histology, and OCT from baseline to 5-year follow-up in patients treated with biodegradable vascular scaffolds. Seventy-two IVUS–virtual histology and OCT paired matched cross-sectional in-and-out-scaffold segments were fused at baseline and follow-up. In total, 46 calcified plaques at follow-up were detected using the fusion method (33 in-scaffold, 13 out-scaffold), showing either calcium progression (52.2%) or de novo calcifications (47.8%). On OCT, calcification volume increased from baseline to follow-up by 2.3 ± 2.4 mm³ (p=0.001). The baseline virtual histologic tissue precursors of dense calcium at follow-up were necrotic core in 73.9% and fibrous or fibrofatty plaque in 10.9%. In 15.2%, calcium was already present at baseline. Precursors on OCT were lipid pool in 71.2%, fibrous plaque in 4.3%, and fibrocalcific plaque in 23.9%. Thus, necrotic core is the most frequent precursor of calcification.

(4) Statins and Coronary Plaque Calcification

Statin therapy effectively reduces cardiovascular events in the settings of primary and secondary prevention. The intracoronary imaging studies using IVUS have clearly demonstrated that high-intensity statin therapy results in atheroma regression in patients with stable, non-obstructive coronary artery disease. Coronary artery calcification (CAC) has been strongly established as an independent predictor for adverse cardiovascular events and progression of CAC has been associated with a higher rate of events. Since CAC reflects a linear estimate of the overall plaque burden of coronary artery atherosclerosis, it has been assumed that statin therapy reduces CAC in parallel with regression of atheroma volume. However, a recent meta-analysis including 8 pooled clinical trials to evaluate the effects of intensive statin therapy on the percent atheroma volume as assessed by serial IVUS imaging showed that high-intensity statin therapy significantly promotes coronary plaque calcification despite regressing atheroma volume. Several other recent studies showed similar results. Therefore, statin may stabilize coronary artery disease through its dual actions on plaque morphology, that is, regression of atheroma and increment of calcified deposit.

The stimulatory effects of statins on plaque calcification may be related to its anti-inflammatory action on atherosclerotic lesion. A recent prospective multicenter study examining the impact of statins on coronary artery inflammation using 18F-fluorodeoxyglucose (FDG) PET/CT showed that atorvastatin treatment for 12 weeks reduced 18F-FDG uptake more potently in the plaque lesions with high-risk morphology. Statins exert their anti-inflammatory effects on the vascular wall through a variety of molecular pathways of the innate and adaptive immune systems, their impact on the circulating levels of pro-inflammatory cytokines, and their effect on adhesion molecules. Interestingly, atorvastatin promotes human monocyte differentiation towards alternative M2 macrophages through p38 mitogen-activated protein kinase-dependent peroxisome proliferator-activated receptor-γ activation. Furthermore, simvastatin accelerates wound
healing in diabetic mice by enhancing repair responses in association with M2 polarization of infiltrated macrophages in granulation tissue. Therefore, statins may facilitate the healing process against plaque inflammation by enhancing M2 polarization of macrophages, resulting in increased plaque calcification as well as plaque regression (Fig. 1). In other words, statins may promote 'anti-inflammation-driven macrocalcification', but not 'pro-inflammation-driven microcalcification' within atherosclerotic plaque.

**Conclusion**

Plaque calcification is an inflammation-dependent process observed in all phases of atherosclerosis. Macrophages can undergo two distinct polarization states, that is, pro-inflammatory M1 phenotype in progression and anti-inflammatory M2 phenotype in regression. In plaque progression, M1 macrophages promote microcalcification, while in plaque regression M2 macrophages may facilitate macrocalcification. OSM may play a pivotal role in the development of plaque calcification, since it has been demonstrated to promote osteoblast formation in bone. Moreover, OSM may induce M2 polarization of macrophage phenotype. Therefore, OSM may play a central role in the development of anti-inflammatory macrocalcification as well as pro-inflammatory microcalcification.

Statin therapy effectively reduces cardiovascular events in the settings of primary and secondary prevention and may stabilize coronary artery disease through its dual actions on plaque morphology, that is, regression of atheroma and increment of calcified deposits. Statins may facilitate the healing process against plaque inflammation by enhancing M2 polarization of macrophages, resulting in increased plaque calcification as well as plaque regression. However, the precise mechanisms inducing macrocalcification within plaque lesions by M2 macrophages remain unclear. Further studies are necessary to clarify this issue.

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**Conflict of Interest**

None

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