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Small entities with large impact: microcalcifications and atherosclerotic plaque vulnerability

Joshua D. Hutcheson, Natalia Maldonado, and Elena Aikawa

Purpose of review

Atherosclerotic plaque rupture and subsequent acute events, such as myocardial infarction and stroke, contribute to the majority of cardiovascular-related deaths. Calcification has emerged as a significant predictor of cardiovascular morbidity and mortality, challenging previously held notions that calcifications stabilize atherosclerotic plaques. In this review, we address this discrepancy through recent findings that not all calcifications are equivalent in determining plaque stability.

Recent findings

The risk associated with calcification is inversely associated with calcification density. As opposed to large calcifications that potentially stabilize the plaque, biomechanical modeling indicates that small microcalcifications within the plaque fibrous cap can lead to sufficient stress accumulation to cause plaque rupture. Microcalcifications appear to derive from matrix vesicles enriched in calcium-binding proteins that are released by cells within the plaque. Clinical detection of microcalcifications has been hampered by the lack of imaging resolution required for in-vivo visualization; however, recent studies have demonstrated promising new techniques to predict the presence of microcalcifications.

Summary

Microcalcifications play a major role in destabilizing atherosclerotic plaques. The identification of critical characteristics that lead to instability along with new imaging modalities to detect their presence *in vivo* may allow early identification and prevention of acute cardiovascular events.

Keywords

microcalcification, plaque rupture, vulnerable atherosclerotic plaque

INTRODUCTION

Clinical studies show that calcium score is an excellent predictor of cardiovascular morbidity and mortality, and coronary calcification is the most widely used marker of the advancement of atherosclerosis [1,2]; however, the link between calcification and plaque rupture is still controversial [3^{*}]. Moreover, the identification of atheromas prone to rupture and cause subsequent acute cardiovascular events, such as myocardial infarction and stroke, is still challenging.

Formerly, the prevailing view was that the presence of calcification within atherosclerotic plaques acted as a biomechanical stabilizer. Indeed, large calcifications easily detected with coronary computed tomography (CT) do not seem to increase plaque vulnerability [4]. However, recent studies indicate an inverse relationship between cardiovascular risk and calcification density [5^{**}], and spotty or speckled areas of calcification, that can be observed in intravascular ultrasound (IVUS) [6] or optical coherence tomography (OCT) [7] are a

good indicator of susceptibility of rupture [8]. These observations provide insights into the role calcification may play in the stability of the atherosclerotic plaque and suggest that it is not only the amount of vascular calcification, but the morphology, size and location that affect plaque vulnerability. A recent biomechanical explanation for the contribution of low density, spotty calcifications to plaque rupture is

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KEY POINTS

- Calcification is a significant predictor of cardiovascular morbidity and mortality.
- Microcalcifications in the fibrous cap of atherosclerotic plaques lead to considerable stress accumulation destabilizing the plaque.
- Emerging imaging modalities may be used to predict the presence of microcalcifications before plaque rupture, potentially identifying patients at risk for myocardial infarction and stroke.

centered on the presence of small microcalcifications that exist within the thin fibrous cap of atherosclerotic plaques [3[•],9–11,12^{••}].

In this review, we will focus on the biomechanical mechanisms by which microcalcifications contribute to plaque instability with special emphasis given to the important characteristics of dangerous microcalcifications. We will then discuss our current understanding of the formation of microcalcifications. Finally, we will discuss emerging imaging techniques that have the potential to identify dangerous microcalcifications forming within atherosclerotic fibrous caps in order to inform clinical decisions prior to an acute vascular event.

MICROCALCIFICATIONS AND PLAQUE RUPTURE

The fibrous cap that overlies the soft necrotic core characteristic of the fibroatheroma [13] is likely to rupture depending on its mechanical stability. If the tissue stress in the cap exceeds a critical peak circumferential stress (PCS), a vulnerable plaque will rupture at the location in which the stress is maximum [14]. Inflammation, metalloproteinases, macrophage infiltration and cell apoptosis affect mechanical properties of the tissue and result in reduced cap thickness, increased core size and abnormal tissue composition, all leading to increased stress in the fibrous cap of the atheroma [15,16].

Several biomechanical models relate PCS to tissue properties, plaque morphology, cap thickness and necrotic core size, based on the principle that the biomechanical stability of an atheroma determines its vulnerability. Numerical studies using initially two-dimensional [13] and, more recently, three-dimensional finite element analyses [5^{••}] and fluid-structure interaction models [17] indicate that local tissue properties significantly modify plaque stability. However, these criteria are insufficient to explain almost 40% of the ruptures, suggesting that other unforeseen factors may play an important role in

distinguishing a lesion prone to rupture from a stable one.

A plausible link between calcification and plaque rupture came with the detection of minute microcalcifications less than 60 μm size embedded right in the fibrous cap of human atheromas [9,18]. The presence of hard inclusions, as microcalcifications, in a much softer hyperelastic layer, the fibrous cap, creates a mismatch in tissue properties and large stress concentrations at the interface between cap and microcalcifications [14], and can lead to sudden rupture of the fibrous cap [9]. This stress concentration effect depends mostly on microcalcification size, along with its location, composition, shape and proximity to other microcalcifications [11,19]. As discussed in the following section, the origin of these dangerous microcalcifications may be the aggregation of calcified matrix vesicles [12^{••}], repeatedly found in human atheromas [20–23]. Even though matrix vesicles, approximately 50–300 nm, initially don't seem to significantly affect plaque vulnerability (an estimated 35% increase in PCS [24]), it is the aggregation of matrix vesicles that form progressively larger microcalcifications, eventually reaching a critical size, 5–60 μm [11], that can trigger the rupture of the fibrous cap by increasing local stresses more than 500% [12^{••}]. Figure 1 shows how microcalcifications observed in both mice (Fig. 1a) and human atheromas (Fig. 1b) have a distinct shape likely due to aggregation of smaller particles and how their presence affects the stress distribution in the cap (Fig. 1c, d).

ORIGIN OF MICROCALCIFICATIONS

The origin of microcalcifications remains largely unknown; however, recent evidence suggests that matrix vesicles released by cells within the plaque may serve as nucleating foci for the formation of microcalcifications [17,25[•],26^{••},27]. Matrix vesicles have long been observed in the development of bone, wherein the vesicles are released by living resident cells and are enriched in calcium-binding proteins that serve as initiating sites for nucleation of calcium phosphate crystals [28–30]. Bone-derived matrix vesicles have also been shown to contain the enzyme alkaline phosphatase, which converts pyrophosphate into free phosphate ions [31,32]. Mineral has been proposed to form within the matrix vesicles as calcium sequestered within the vesicle membrane and the phosphate generated by the activity of alkaline phosphatase reach sufficient nucleating concentrations [33]. The resultant hydroxyapatite mineral that forms from this process is then deposited along collagen fibers within the

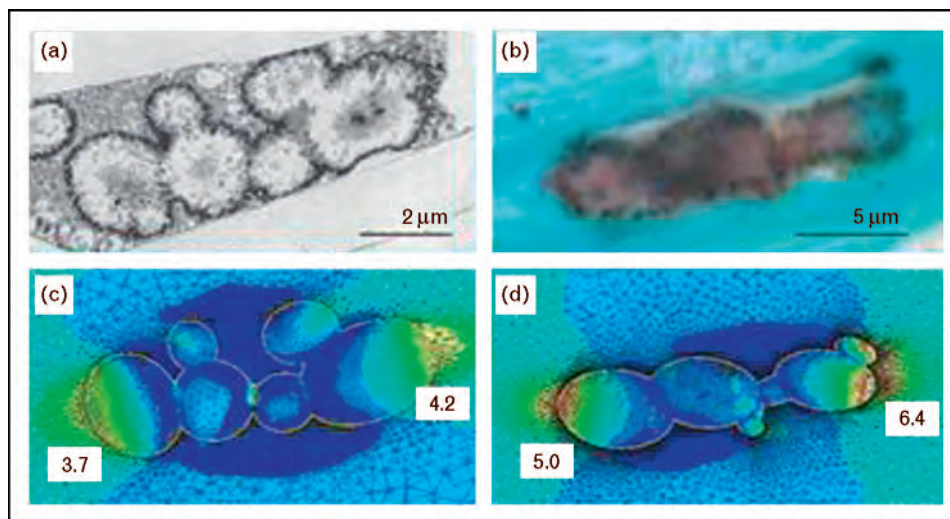


FIGURE 1. Transmission electron microscopy and histology-based finite element analyses. (a) Transmission electron microscopy image of aggregated calcifying matrix vesicles forming microcalcifications in a mouse atheroma. (b) Image of a microcalcification embedded in a human fibrous cap, obtained from nondecalcified histology, and stained with von Kossa. (c and d) Stress distribution at the interface of the microcalcifications in a and b, respectively, assuming that they are embedded in fibrous caps under tension. The numbers indicate the factor by which stress is increased and concentrated at the poles of the microcalcifications. Reproduced with permission from [12²²].

developing bone [30]. Vascular calcification has been proposed to involve similar processes, whereby pathologic conditions cause calcifying matrix vesicles to be released from cells within the vascular wall [25²¹]. Studies have indicated that both vascular smooth muscle cells [17] and macrophages [26²²] have the propensity to release calcifying matrix vesicles, and matrix vesicles have been observed in calcific regions within human and mouse atheromas [20]. Corresponding with the observation that cholesterol and matrix vesicles colocalize within atherosclerotic lesions (Fig. 2a), oxidized forms of cholesterol have been shown to enhance the calcifying activity of matrix vesicles isolated from rabbit aortae [34].

In contrast to bone matrix vesicle processes, in which mineralization is alkaline phosphatase dependent, *in-vitro* studies indicate that vascular calcification may involve both alkaline phosphatase dependent and independent matrix vesicle-induced mineralization processes [17,25²¹]. When macrophages or smooth muscle cells are stimulated with the addition of phosphate ions, vascular matrix vesicle mineralization mechanisms appear to be independent of alkaline phosphatase; smooth muscle cell-derived matrix vesicles have been shown to require the calcium-binding protein annexin A6 for calcification [17], whereas mineralization of macrophage matrix vesicle calcification appears to involve a complex of annexin A5 and another calcium-binding protein, S100A9 [25²¹]. When phenotypic switching of smooth muscle cells to an

osteogenic phenotype is achieved *in vitro* using a common osteogenic milieu, it involves cell differentiation and expression of alkaline phosphatase to hydrolyze phosphoric acid monoesters into free phosphate ions [35]. *In vivo*, osteogenic reprogramming of vascular smooth muscle cells has been observed [35]; however, as alkaline phosphatase activity generates free phosphate ions, nonosteogenic vesicle populations may serve as additional calcifying foci.

Using advanced microscopic analyses, a recent study demonstrated the pervasiveness of calcifying spherical particles throughout excised human cardiovascular tissues [36²³]. The identified spherical structures ranged from the size of dangerous microcalcifications ($\sim 5 \mu\text{m}$) down to the size of individual matrix vesicles ($\sim 100 \text{nm}$). The detailed progression of mineralization from the nucleating events within matrix vesicles to the development of dangerous microcalcifications is still uncertain because of inadequate techniques to monitor these processes *in vivo*. Further, the differences between large, plaque stabilizing calcifications and dangerous microcalcifications remain unknown. Matrix vesicles may serve as nucleating foci for both types of calcification with the major difference being the aggregation and location of the vesicles (Fig. 2b). Future works focusing on this progression may give new insight into the controllability of this process for clinical intervention and early detection methods that can be used to predict plaque vulnerability.

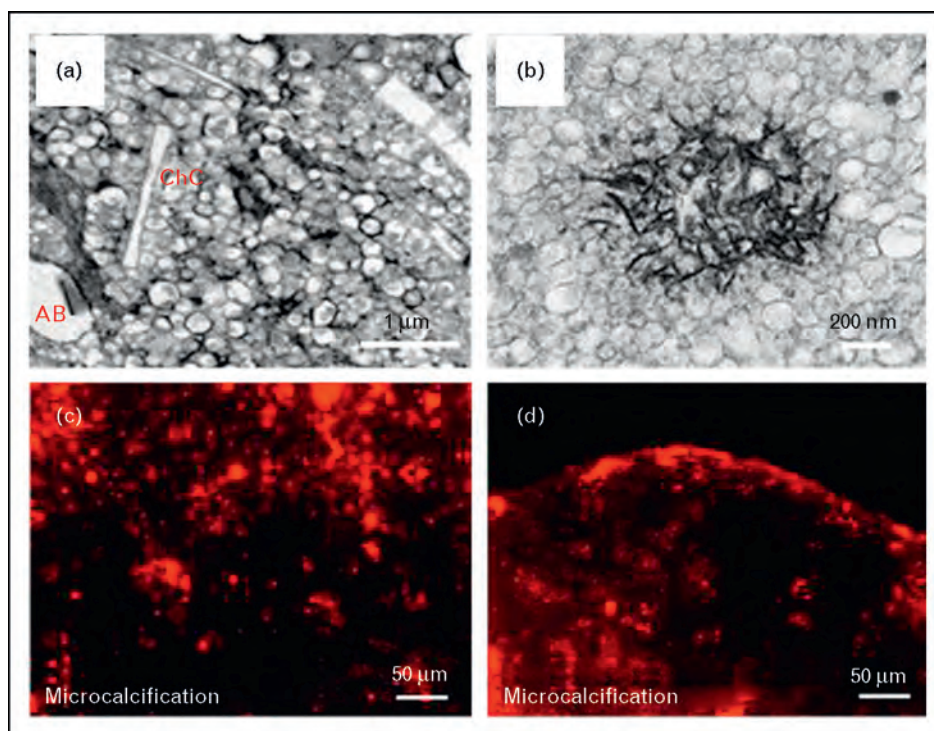


FIGURE 2. Matrix vesicles and microcalcifications in atherosclerotic plaques. (a) Transmission electron microscopy image of matrix vesicles aggregating within a plaque in close proximity to ChC. An AB is shown for size comparison. (b) Transmission electron microscopy of aggregating matrix vesicles nucleating mineralization. (c) NIRF staining of microcalcifications within a mouse plaque. (d) NIRF staining of microcalcifications at the plaque border. AB, apoptotic body; ChC, cholesterol crystals; NIRF, near-infrared fluorescent. Reproduced with permission from [26**].

DETECTION OF MICROCALCIFICATIONS AND CLINICAL IMPLICATIONS

In order for our increased understanding of the significant contribution of microcalcifications to atherosclerotic plaque vulnerability to impact clinical decisions, imaging modalities must first be developed to identify the presence of dangerous microcalcifications prior to plaque rupture. The small size of microcalcifications presents a major challenge for imaging modalities to detect potential regions of vulnerability *in situ*. Calcification is traditionally imaged using CT, which can give an accurate measure of overall calcium burden, and improved risk prediction is possible through the identification of spotty calcifications with IVUS or OCT. However, CT lacks the resolution to identify specific dangerous microcalcifications within arterial walls [37], IVUS requires an invasive catheterization procedure, and standard OCT is limited by tissue penetration depth. Recent advancements using PET/CT with ^{18}F -sodium fluoride (^{18}F -NaF), an established PET tracer for bone formation and remodeling, may provide new strategies for honing in on regions of plaque vulnerability [38]. Coronary uptake of ^{18}F -NaF was found overlaying, adjacent to and distal from regions of CT identified

calcifications [39]. Additionally, large areas of calcification with no ^{18}F -NaF uptake were observed. This suggests that, as with bone, ^{18}F -NaF uptake in the vasculature is a marker of ongoing calcific remodeling [39]. Large, stable calcifications do not exhibit ^{18}F -NaF uptake, whereas active regions of biomineralization accumulate ^{18}F -NaF. The ^{18}F -NaF signals far away from the CT identified calcific regions may represent the dangerous microcalcifications that cannot be detected by traditional imaging modalities [39]. In support of this hypothesis, a prospective clinical trial showed high ^{18}F -NaF accumulation in the culprit coronary plaques in cases of myocardial infarction and in ruptured carotid artery plaques [40**]. Histological evaluation of these plaques revealed active calcification processes. These PET/CT techniques exhibit promise in identifying particularly vulnerable regions within the vasculature; however, they still do not have the resolution necessary to identify specific microcalcifications that may contribute to plaque rupture. One strategy may be to use PET/CT imaging to identify potentially vulnerable regions followed by more invasive catheter-based imaging of these regions to assess plaque and calcification morphology. In this way, clinicians may be able to implement the size and

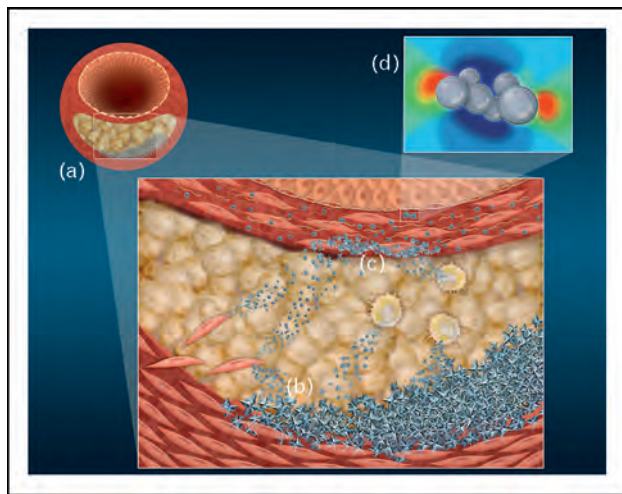


FIGURE 3. Schematic of our current understanding of the vesicle-derived calcification process. (a) Typical cross section of a fibroatheroma with a thin fibrous cap and large calcification. (b) Smooth muscle cells and macrophages release vesicles that contribute to large calcifications and (c) microcalcifications within the fibrous cap. (d) Vesicles accumulating in the fibrous cap form microcalcifications creating stress concentrations as shown by finite element analysis that can lead to cap rupture.

morphology criteria established by biomechanical modeling to make informed treatment decisions.

In addition to the advent of promising techniques to identify vulnerable plaques *in situ*, fluorescent probes already in use offer the resolution to identify microcalcifications in preclinical animal models and pathological analyses of resected tissues (Fig. 2c, d). Near-infrared fluorescent (NIRF) probes for hydroxyapatite, the calcium phosphate-based mineral involved in calcification, allow earlier detection of calcification in human plaques than light microscopy techniques currently used by pathologists [41]. These tracers are based on the conjugation of a NIRF moiety attached to a bisphosphonate backbone. Bisphosphonates have a similar structure to pyrophosphate, irreversibly bind calcium and accumulate in regions of calcium-based mineralization. NIRF tracers have been successfully used in intravital microscopy to detect vascular calcification in mice [41–43]; therefore, this technique can be utilized to monitor the progression of calcification in longitudinal animal studies and diagnose microcalcifications on histological sections. This enhanced resolution due to NIRF signal amplification may allow researchers to understand the nucleation of microcalcifications and allow pathologists to readily identify the presence of microcalcifications in subclinical atherosclerotic plaques. The formation of calcified plaques may also give insight into the

nucleation and aggregation mechanisms that lead to large calcifications.

CONCLUSION

Emerging evidence suggests that microcalcifications play a critical role in determining atherosclerotic plaque vulnerability (Fig. 3). Model-based biomechanical analyses of plaques indicate that the size, morphology, size and location of calcification within the plaque are more important indicators of plaque stability than the presence of calcification solely. The presence of small microcalcifications within the fibrous cap of the plaque can greatly increase the amount of stress in the cap. When this stress exceeds the threshold required for cap failure, the plaque ruptures, leading to thrombus formation and vessel occlusion. The rupture of asymptomatic plaques and vessel occlusion are silent contributors to sudden stroke and myocardial infarction, the leading causes of cardiovascular morbidity and mortality. Studies suggest that vascular calcifications form as part of an active process wherein cellular-derived vesicles within the plaque serve as nucleating foci for the formation of calcium phosphate mineral. This active mechanism provides hope that therapeutic strategies may be developed to target dangerous microcalcifications. As future studies progress our understanding of the nucleating events of mineralization, the development of preventive strategies for calcification or therapeutic interventions that may reduce calcification or shift it to a more favorable phenotype may be within reach.

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Conflicts of interest

There are no conflicts of interest.

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