Role of Extracellular Vesicles in De Novo Mineralization
An Additional Novel Mechanism of Cardiovascular Calcification

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Abstract—Extracellular vesicles are membrane micro/nanovesicles secreted by many cell types into the circulation and the extracellular milieu in physiological and pathological conditions. Evidence suggests that extracellular vesicles, known as matrix vesicles, play a role in the mineralization of skeletal tissue, but emerging ultrastructural and in vitro studies have demonstrated their contribution to cardiovascular calcification as well. Cells involved in the progression of cardiovascular calcification release active vesicles capable of nucleating hydroxyapatite on their membranes. This review discusses the role of extracellular vesicles in cardiovascular calcification and elaborates on this additional mechanism of calcification as an alternative pathway to the currently accepted mechanism of biomineralization via osteogenic differentiation. (Arterioscler Thromb Vasc Biol. 2013;33:1753-1758.)

Key Words: atherosclerosis ■ calcification ■ extracellular vesicles ■ inflammation ■ macrophages ■ rupture ■ valve disease

Cardiovascular calcification, a growing burden in Westernized countries, is not only a risk factor for cardiovascular events, but may itself contribute to cardiovascular risk.1 A growing number of studies have demonstrated that microcalcification in vulnerable plaques contributes to plaque destabilization and fatal plaque rupture.2-5 Calcification of the cardiovascular system, including the coronary arteries and heart valves, follows an active process in which smooth muscle cells (SMCs) or valve interstitial cells undergo osteogenic differentiation.6-8 In addition, it is now evident that calcification progresses through, and indeed may be initiated by, the release of calcifying extracellular vesicles by cells residing in the calcification niche.2,9

Extracellular vesicles possess a metabolically active outer membrane that protects the internal cargo, consisting of proteins, microRNA, and other components from the parental cell. They can be found throughout the body in various tissues and fluids, and they participate in both physiological and pathological processes. Their involvement in a broad range of pathological pathways has made them attractive diagnostic biomarkers,10 whereas their therapeutic use is an emerging field.11,12 Extracellular vesicles seem to have advantages over existing drug delivery systems because of their size, lack of toxicity, and target specificity. A growing number of studies have contributed to the concept that cells implicated in the progression of cardiovascular calcification release active extracellular vesicles capable of nucleating hydroxyapatite.13-15 This emerging subset of the field provides additional mechanisms to therapeutically target cardiovascular calcification.

Discovery of Calcifying Extracellular Vesicles
The discovery of cell-derived extracellular vesicles followed the introduction of transmission electron microscopy in the mid-20th century. The groups of Anderson16 and Bonucci17 discovered that extracellular vesicles associate with the earliest sites of mineral formation in bone and cartilage mineralization. These extracellular membrane-bound structures were later termed matrix vesicles (MVs). Physiological mineralization is now widely believed to be initiated in bone, dentin, and cartilage by vesicles released from specific regions of the outer membranes of osteoblasts, odontoblasts, and osteoclasts.18 Using ultrastructural, histological, and cytochemical techniques, Anderson et al19 showed the presence of MV-like structures that were believed to originate from SMCs.

Classification Controversy
Much controversy exists in this field about the classification and nomenclature used for extracellular vesicles. Depending on size and type, extracellular vesicles are broadly classified as ectosomes (or shedding microvesicles), exosomes, and apoptotic bodies.20 Ectosomes, also known as microparticles, are large extracellular vesicles ranging from 50 to 1000 nm in diameter; exosomes are small membranous vesicles of endocytic origin ranging from 40 to 100 nm in diameter; and apoptotic bodies are released from fragmented apoptotic cells and are 50 to 5000 nm in diameter. MVs (the main focus of this review) are another category that should be added to this classification. MVs are small membranous structures (30–300 nm in diameter) surrounded by a lipid bilayer, produced by blebbing of the plasma membrane, and can calcify. The
Pathological Calcification
Cardiovascular calcification is now recognized as regulated biomineralization that follows similar pathways to that of bone development. Nuclear magnetic resonance and X-ray diffraction techniques have shown that the matrix-mineral atomic interface in calcified plaque is similar to that in bone. In addition to bone-like mechanisms, several studies have indicated that, other mechanisms, including cell death, also contribute to cardiovascular calcification. A combination of in vitro studies and in vivo molecular imaging have formed the concept of an inflammation-dependent calcification paradigm, suggesting that macrophage infiltration and inflammation precede calcification, and activated proinflammatory pathways induce osteogenic transformation of SMCs or release of MVs.

Calcifying extracellular vesicles have been identified in calcifying aortic valves, atherosclerotic intimal lesions, and medial arterial calcification, similar to the MVs involved in physiological bone mineralization. Literature is lacking about the role of MVs in calcifying aortic valves, which could differ from the role of MVs in arterial calcification, although this concept requires further investigation. MVs may initiate the mineralization processes akin to those in bone, and may induce the rupture of vulnerable plaques. During the early stages of calcification, MVs and apoptotic bodies released from macrophages and SMCs may contribute to the calcification process. Regions of spotty calcifications in atherosclerotic lesions that contain calcified vesicles have been predicted, via finite element simulations, to have increased stress levels and to be prone to rupture, which can lead to acute thrombosis and fatal myocardial infarction. Patients with chronic renal disease (CRD) have advanced atherosclerosis and a high cardiovascular mortality rate. The severity and complexity of CRD causes a perfect storm of metabolic dysfunction, leading to accelerated intimal and extensive medial calcification. Concentrations of extracellular calcium and phosphate, similar to those found in serum of patients with CRD on dialysis, induce the release of calcifying MVs from cells involved in cardiovascular calcification, such as vascular SMCs and macrophages. The plaques of non-CRD patients also contain MVs, suggesting that this phenomenon may occur in an atherosclerotic or diabetic milieu.

Structure
Ultrastructural studies of components of the cardiovascular system have identified highly heterogeneous mineral-associated vesicles (MVs) ranging from 30 to 300 nm in diameter and displaying different structural appearances. They are typically double-membrane-bound bodies that are round or ovoid in shape, often associated with extracellular matrix
components, particularly collagen, and displaying evidence of hydroxyapatite crystals on the inner membrane within the lumen, and on the outer membrane of the vesicle (Figure 1).

**Biogenesis**

MVs arise from cells during physiological mineralization through a budding process, originating from specialized regions of the cell plasma membrane and released in the same orientation as that of the membrane. When the vesicle buds from the cell, it takes a subset of the cargo of the cell with it, safely packaged within a region of the membrane. Evidence suggests that the plasma membrane of the parenting cell contains components similar to those of the released vesicle, but in different quantities. MVs are rich in annexins and acidic phospholipids, such as phosphatidylserine, compared with the corresponding plasma membranes, which enable them to calcify.

**MV Cargo**

Extracellular vesicles mediate active communication between cells. They participate in the exchange of functional and genetic information, and in the mediation of adaptive immune responses. The cargo carried by extracellular vesicles dictates their function. Proteomics studies have provided evidence of the cargo contained within osteoblast-derived and chondrocyte-derived MVs. Numerous noncalcifying extracellular vesicles also appear within calcifying plaques. Studies by Kapustin et al and Reynolds et al have provided evidence suggesting that noncalcifying vesicles contain inhibitors of calcification, such as fetuin-A and matrix Gla protein, which decrease in MVs released by cells in a CRD milieu, enabling them to calcify. These MVs may possess a physiological function under nonpathological conditions. In one study, proteomics analysis of vascular SMC-MVs detected signaling molecules. Therefore, calcifying vesicles may be merely dysfunctional vesicles, released to act as intercellular communicators. However, cells may release these particular vesicles as a reaction to stress, as a means of releasing unwanted cargo, such as excess calcium.

**Nucleation Core**

Phosphatidylserine forms a complex with calcium at sites of early mineralization in skeletal tissue, and inorganic phosphate is required to form the nucleation core. We now believe that in both physiological and pathological MVs, annexins also may contribute to the formation of nucleation complex. Annexins, specifically annexin-2, -5, and -6, are major proteins within MVs. They facilitate calcium influx and mineralization by binding to phosphatidylserine and forming ion channels in the MV membrane. The exact importance of annexins in MV mineralization, however, is still under investigation.

Annexin-5 was previously shown to facilitate nucleation and growth of mineral. Despite this finding, a recent in vivo study produced evidence that a lack of annexin-5 and annexin-6 functionality does not affect skeletal development in mice. These results suggest that a compensatory mechanism is at play, another member of the annexin family may compensate for the loss of annexin-5 and annexin-6. Annexin-5 is seemingly important in the mineralization potential of macrophage-derived

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**Figure 1.** Mechanism of pathological matrix vesicle mineralization: a mineral imbalance leads to an influx of phosphate and calcium into the vesicle via appropriate channels, alkaline phosphatase generates inorganic phosphate in the extravascular space. Nucleation of hydroxyapatite (HA) is facilitated by an annexin (Anx)-phosphatidylserine (PS) complex, enabling generation of microcalcification/calcification. CRD indicates chronic renal disease; and SMC, smooth muscle cells.
MVs involved in biomineralization.\textsuperscript{35} In contrast, annexin-6 seems to play a main role in SMC-MV mineralization.\textsuperscript{14} The nucleation core is required for nucleation, a physiochemical process through which ions accumulate with the correct orientation to mimic a crystal surface, to ensue. Recently, we demonstrated that proinflammatory and prothrombotic S100A9 might be involved in nucleation of MVs via formation of phosphatidylyserine–annexin-5–S100A9 complex.\textsuperscript{15}

**Nucleation, Crystallization, and Microcalcification Formation**

Whether crystal formation or transdifferentiation of vascular SMCs occurs first is debatable.\textsuperscript{50} Whether osteogenic transformation of SMCs is required for MV release and calcification is also unknown. But we do know that in skeletal tissue, the first crystals of hydroxyapatite are formed within extracellular vesicles. This phenomenon can be split into 2 phases: in conditions of mineral imbalance, the influx of calcium and phosphate into the extracellular vesicles, via their appropriate channels, leads to initial mineral accumulation in the form of amorphous calcium phosphate.\textsuperscript{51} For mineralization to proceed, the levels of phosphate and pyrophosphate must be in balance,\textsuperscript{52} achieved via alkaline phosphatase within the membranes of calcifying extracellular vesicles in bone.\textsuperscript{53} Whether MVs involved in cardiovascular calcification contain alkaline phosphatase, however, is controversial, with conflicting reports on alkaline phosphatase within vascular SMC–derived MVs.\textsuperscript{13,14} The second phase, mineral propagation, seems to ensue via the release of crystal through the MV membrane, exposing preformed hydroxyapatite nanocrystals to the extracellular fluid. Once exposed to this fluid, the nanocrystals can act as loci or templates for the formation of new crystals via homologous nucleation,\textsuperscript{9} and perhaps mineralize extracellular matrix components. We presume that MVs associated with pathological ectopic calcification generate crystals in a similar way.

MVs bind to extracellular matrix components, such as collagens and glycosaminoglycans. Vesicles seem to be the initial site of nucleation, and intravesicular mineral crystals seed these extracellular matrix components. Vesicle–glycosaminoglycans interaction is enhanced in mineralization-competent/calcifying chondrocyte-derived MVs.\textsuperscript{34} Atherosclerotic plaque contains a predominance of glycosaminoglycans associated with mineralization similar to that of bone, further suggesting that glycosaminoglycans play a direct role in initiating pathological and physiological mineralization.\textsuperscript{27} Crystalline extracellular vesicles and calcified collagen fibers that abound in Randall plaques (found in renal medulla) seem to follow this alternative mechanism of MV mineralization, akin to vascular calcification.\textsuperscript{55}

Molecular imaging has demonstrated that inflammation and microcalcification evolve within close proximity in intimal arterial calcification, and overlap at border regions that are prone to rupture.\textsuperscript{33} A positive feedback loop of calcification and inflammation drives disease progression.\textsuperscript{56} Calcified extracellular vesicles/microcalcification in vulnerable macrophage-rich plaques may contribute to plaque destabilization and rupture, as predicted by Vengrenyuk et al.\textsuperscript{2–4} Components of calcifying extracellular vesicles, including prothrombotic tissue factor and S100A9, may further lead to acute cardiovascular events. A similar inflammation-driven mechanism seems to ensue in calcific aortic valve disease.\textsuperscript{33,38} Although our electron microscopy observations show that MVs closely associate with microcalcification, suggesting a role of calcifying MVs in the generation of microcalcification (Figure 2), the exact mechanism of pathological microcalcification formation by MVs remains to be elucidated.

**Conclusion**

Much of our knowledge of the role of extracellular vesicles in cardiovascular calcification relies heavily on previously established evidence of MVs involved in physiological bone mineralization. Although research in this field dates back to the 1970s, the role of extracellular vesicles in pathological calcification is still largely unknown. Progression of this
field has been hindered by the lack of sophisticated modalities to visualize extracellular vesicles in vitro and in vivo. Novel modalities that can detect the size and numbers of extracellular vesicles and visualize them in vitro in real time, such as nanoparticle tracking analysis, are emerging. In addition, molecular imaging has proven useful in visualizing fluorescently probed calcifying vesicular structures in vivo in live animals. The development of innovative technologies would further advance this field of research. Further understanding of extracellular vesicle structure and function may pinpoint novel means of treating diseases associated with these entities.

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References
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