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Review

Extracellular vesicles in vascular calcification

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ABSTRACT

Vascular calcification is associated with adverse cardiovascular events that increase the risk of cardiovascular death. Unfortunately, the pathogenesis of vascular calcification is complex and incompletely understood. As important intercellular signaling molecules, the role of extracellular vesicles (EVs) in vascular calcification has attracted wide attention in recent years. This review will briefly describe the role of EVs (mainly including exosomes and microvesicles) in the process of vascular wall calcification focusing on the specific mechanisms of smooth muscle cell (SMC) differentiation and calcium-phosphorus balance to illustrate the relationship between EVs and vascular calcification. It is likely that EVs may be prognostic markers in some cardiovascular diseases and have potential therapeutic potential.

1. Introduction

Vascular calcification is abnormal mineralization that occurs in the cardiovascular system resulting in reduced elasticity of the vessel wall and impaired vascular structural integrity. Vascular calcification is a common in diabetes, calcific aortic valve disease, chronic kidney disease (CKD) and chronic inflammatory disease that substantially increases the incidence and mortality of hypertension, aortic stenosis, myocardial hypertrophy, myocardial ischemia and congestive heart failure. Vascular calcification can occur in the media and intima of almost all arteries [1]. The clinical manifestations of vascular calcification depend on the location within the arterial wall and the perfused tissue, with coronary artery calcification being most pronounced. Intimal calcification of the coronary arteries can lead to coronary artery stenosis and acute thrombosis or coronary ischemia [2]. With the prolongation of human life span, the incidence of vascular calcification has gradually increased. At present, vascular calcification is one of the most common and refractory diseases, and there is still no effective treatment for vascular calcification in clinic [1].

In recent years, studies have found that vascular calcification is an active, regulatable biological process similar to bone formation [3]. The cause of vascular calcification is complex, and there are many possible mechanisms, including vascular smooth muscle cells (VSMCs) instability, calcium and phosphate and elastin degradation, osteoclast differentiation and apoptosis [4]. Evidence from new calcification mechanisms suggests that active EVs derived from SMCs, stromal cells, and macrophages are important mediators of plaque calcification in heart valves and atherosclerosis (AS). Ultrastructural analysis revealed that EVs existed in calcified human aortic valves, aortic media and AS intimal plaques, The EVs released by SMCs and macrophages in the vessel wall interact with fibrillar collagen to form early calcified mineral crystals [5]. EVs may promote the occurrence and progression of AS by promoting initial injury formation, intravascular calcification, unstable plaque progression and post-rupture thrombosis. In cardiovascular diseases, the number and surface markers of EVs released by different cells are different, which can be used as biomarkers for diagnosis and prognosis. EVs have the characteristics of small size, easy penetration of biofilm, low immunogenicity of lipid bilayer membrane structure and so on. It has the potential to play a therapeutic role as a drug or drug carrier [6].

Abbreviations: EVs, extracellular vesicles; SMCs, smooth muscle cells; CKD, chronic kidney disease; VSMCs, vascular smooth muscle cells; AS, atherosclerosis; MVs, matrix vesicles; ECM, extracellular matrix; GRP, Gla-rich proteins; MGP, matrix GLA protein; MAPK, mitogen-activated protein kinase; NOX, NADPH oxidase; DDR-1, discoidin domain receptor-1; TNAP, tissue non-specific alkaline phosphatase; ECs, endothelial cells; Cbf, core binding factor; ALPL, alkaline phosphatase; smpd-3, Sphingomyelin phosphodiesterase-3; BMP-2, bone morphogenetic protein-2; TNF-α, Tumor Necrosis Factor-α; IL-1β, interleukin-1β; EMPs, endothelial microparticles

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2. Classification and formation of EVs

EVs are microcapsule vesicles secreted by cells that are widely distributed in biological fluids. They are composed of a lipid bilayer membrane with a transmembrane protein and a core. The inner core usually carries soluble proteins, lipids and RNA (Non-coding RNAs such as mRNA, miRNA, LncRNA, and circRNA). In recent years, academic research hotspots include exosomes, microvesicles (formerly known as microparticles) and apoptoticbodies. These research hotspots are all in the category of EVs. As the term to describe EVs hasn't been standardized, oncosomes, argosomes, epididimosomes, dexosomes, microvesicles, prostasomes, exosome-like vesicles, texosomes, archaeosomes and promininosomes were used to discribe EVs based on the source of isolation [7]. Academic circle tend to classfied EVs based on the mechanism of biogenesis rather than the source of isolation currently. In this way, EVs can be divided into three types, namely exosomes, microvesicles and apoptotic bodies.

Exosome was initially discovered by Dr. Rose Johnstone to understand the biologic process of a reticulocyte transform to a mature erythrocyte [8]. Exosomes are formed in the endosomal network, the endosomes can be further divided into three different chambers: early, late, and recycled endosomes. Early endosomes fusion with endocytic vesicles and the inclusion of their contents used for recycling, degradation, or exocytosis [9]. The contents used for recycling are classified into the recycling endosomes. Then, the rest of the early endosome become late endosomes through a series of transformations [9]. During the transformation period, the contents to be degraded or exported are first sorted into the vesicles of 30-100 nm, and the vesicles enter the cavity of the late endosome. These late endosomes concluding multiple small vesicles also known as multi-vesicular bodies (MVBs) [10]. The late endosomes are targeted for fusion with lysosomes or plasma membranes. Fusion with lysosomes will lead to the destruction of the contents of late endosomes. Besides, fusion with plasma membrane release 30-100 nm vesicles to the outer space of cells. These excreted vesicles are exosomes [9]. Microvesicles arise through direct outward budding and fission of the plasma membrane, and is also the result of dynamic interaction between phospholipid redistribution and cytoskeletal protein contraction. Although the size of Microvesicles tend to be larger (50–2000 nm) compared to exosome, the biogenensis is the main difference between microvesicles and exosomes [9]. Apoptotic bodies are diameter of 50–5000 nm and released from fragmented apoptotic cells. Matrix vesicles (MVs), another category should be added to this classification, are diameter of 30–300 nm and small membranous structures surrounded by a lipid biolayer. They are produced by blebbing of plasma membrane, and can calcify [11].

EVs are extracellular biological information carriers that transfer a variety of functional transcripts and lipids to target cells, causing transient or persistent receptor cell phenotypic changes. Different kinds of cells secrete EVs into body fluids and various microenvironments by paracrine and other methods, and play a role in adjacent regions or distant cells [12]. Pathological EVs originating from SMCs, stromal cells and macrophages play an important role in vascular calcification [13]. EVs are involved in physiological and pathological processes such as inflammation, proliferation, thrombosis and vasoactive reactions, and are also involved in the development of AS lesions and promote vascular calcification [6].

3. The role of EVs in vascular calcification

Vascular calcification is a prominent feature of chronic inflammatory diseases such as chronic kidney disease, type 2 diabetes, and AS. It is a biological process involving VSMCs, endothelial cells (ECs), and inflammatory cells. Bone remodeling and vascular calcification have similar pathways, but the exact mechanism of calcification remains unclear. EVs play an important role as regulators in the cardiovascular system and bone biology [14]. In a normal environment, vascular cells release EVs and exert physiological functions to maintain homeostasis. However, under pathological conditions, certain vesicles acquire calcification potential due to changes in cytoskeletal orientation or damage during vesicle transport and loading [13].

3.1. EVs regulate smooth muscle differentiation to promote vascular calcification

Contractile VSMCs maintain vascular tone and cause phenotypic transformation and proliferation after injury, that is, from contractile to synthetic [15]. SMC transdifferentiation into osteoblast-like cells can form bone matrix and promote vascular calcification [16].

Under physiological conditions, most VSMCS showed contractile phenotype, contractile VSMCs actively secrete MVs to regulate microenvironment [17]. MVs are a subgroup of EVS, which are coated in double membranes and consist of phosphatidylserine and annexin [11]. MVs contain endogenous calcification inhibitors such as vitamin K-dependent matrix GLA protein (MGP) and circulating fetuin-a [17]. Under Long-term stress and mineral imbalance, VSMCs can transform synthetic phenotype, promote the release of MVs, and transform them into a calcified state [17]. Calcified EVs released by VSMCs are the smallest molecules to form microcalcification [18]. It is revealed by Materials science imaging techniques that SMC-derived calcifying EVs tend to aggregate and form microcalcifications in areas with sparse collagen when released into the extracellular matrix (ECM) [18]. Large calcification formed by the accumulation of microcalcification and gradually formed mature minerals. The microcalcification formed in the fiber cap of vulnerable atherosclerotic plaques further strengthens the rupture of plaques, while the large calcification under the stable fiber cap can promote the stability of plaques [18]. Collagen acts as a scaffold to guide the shape and size of calcification produced by this growth process [18]. In a hyperphosphatemic environment (e.g., CKD) or inflammation-driven AS, vascular SMCs release EVs enriched with procalcific biomarkers [19]. In patients with CKD, MVs contain less fetuin-A or Gla-rich proteins (GRP), and such MVs were shown to be related to higher severity of mineral calcification in soft tissue. In addition, the EVs in the serum of CKD patients were found to be prone to vascular calcification because they carried a higher percentage of calcification-associated markers, such as GRP [20]. It induce calcification of recipient VSMCs may through multiple signaling pathways such as mitogen-activated protein kinase (MAPK) and NADPH oxidase (NOX) signaling [21]. In atherosclerotic plaques, collagen receptor discoidin domain receptor-1 (DDR-1) deficient SMCs showed a large number of calcified EVs release and deposition of collagen and mineral [22]. At this time, the proteins in calcified EVs have the functions to uptake of $Ca2+$ and inhibit fetuin-a activity in ectopic mineralization [17]. Calcified EVs contain dysfunctional miRNAs could induce gene expression of osteogenic markers such as runx2, Smad1, osterix, tissue non-specific alkaline phosphatase (TNAP), and pro-inflammatory factors [19]. In hyperphosphatemic and osteogenic conditions, fetuin-A and matrix Gla protein decrease significantly. TNAP, runx2, Smad1, osterix which promote extracellular mineralization on the other hand, increased significantly [19]. The formation mechanism of calcified EVs has also aroused widespread interest among researchers. For example, A special transporter, sortlin, may play a key role in it. Sortilin localizes to human calcified blood vessels and mouse AS lesions, which regulates TNAP entry into EVs, thereby increasing the calcification potential of EVs [23] (Fig. 1).

3.2. EVs regulate vascular calcification through calcium-phosphorus balance

Phosphate and calcium play an important role in vascular calcification. High concentrations of phosphate and calcium promote vascular calcification, including stimulation of osteoblast or chondrocyte differentiation, vesicle release, apoptosis, and degradation of ECM [24].

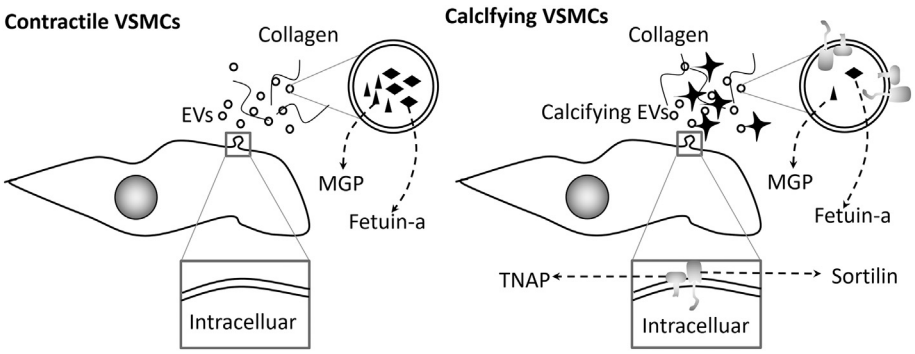

Fig. 1. VSMCs can transform synthetic phenotype, promote the release of EVs, and transform them into a calcified state. Calcifying EVs contain less fetuin-A or Gla-rich proteins (GRP), It tend to aggregate and form microcalcifications in areas with sparse collagen when released into the extracellular matrix (ECM). Sortlin regulate TNAP entry into EVs to increase the calcification potential of EVs.

High concentrations of phosphate accelerate the precipitation of calcium and phosphate in the form of hydroxyapatite. On the one hand, High phosphoric acid (Pi > 2.4 mmol/L) can directly stimulate VSMCs to express core binding factor (Cbf) -a1, which induces phenotypic transformation of VSMCs into osteoblasts [25]. On the other hand, high extracellular phosphate levels repress the production of calcification inhibitors and promote the release of EVs lacking these inhibitors, but with the increased of pro-calcific proteins such as tissue-nonspecific alkaline phosphatase (ALPL). These could form microcalcifications and serve as a nidus for calcium phosphate precipitation and growth of calcium phosphate crystals [26]. In mouse VSMCs, the formation of EVs lacking these inhibitors connect with an osteoblastic transcription factor—osterix. The expression of osterix is stimulated by elevated phosphate levels [27]. Studies have shown that mouse aortic SMCs respond to elevated levels of phosphorus by increasing the release of EVs and altering their protein composition when exposed to high concentrations of phosphate [28]. In VSMCs, the sodium-phosphorus co-transporter PiT-1 promotes matrix calcification caused by elevated phosphorus, while PiT-2 inhibits its changes [4]. In addition, phosphate may limit the affinity of MGP for hydroxyapatite crystals, resulting in relief of vascular calcification [29].

Both in vitro and in vivo studies have shown that calcification is associated with high calcium levels, which are both independent and synergistic with elevated phosphate levels [30,31]. A slight increase in the concentration of Ca2+ significantly increases calcification as the phosphorus concentration increases. Calcification of Ca2+ can trigger calcification of cytoplasmic vesicle nucleation and apoptosis through different pathways, including promotion of VSMCs apoptosis and vesicle release [30].A study shows that vessel rings from healthy control subjects and CKD patients had different reaction with long-term exposure to elevated calcium and/or phosphate. Vessel rings from healthy control subjects did not accumulate calcium. However, predialysis and, to a much greater extent, dialysis vessels did. This suggests that normal VSMCs possess intact inhibitory pathways that prevent calcification [32]. For predialysis and dialysis patients, although high phosphorus increased vascular calcification, Ga elevation was a more effective calcification stimulant for fixed Ga*P [32]. Further studies found that Calcification in CKD vessels was also associated with increased deposition of VSMC-derived vesicles. Electron microscopy confirmed increased deposition of vesicles containing crystalline calcium and phosphate in the extracellular matrix of dialysis vessel rings. In contrast, vesicle deposition and calcification did not occur in normal vessel rings, but extensive intracellular mitochondrial damage was observed [32]. Vesicle release is thought to be an adaptive response, because vesicles extrude Ca from the cell, providing protection from intracellular Ca overload. It suggests that the release of EVs may be initially an adaptive response to release intracellular Ga overload and vessel calcification. Over time, EVS turned into calcified EVs with the depletion of calcification inhibitors and promote the formation of calcification [32]. Sphingomyelin phosphodiesterase-3 (smpd-3) is a key protein in the production of EVs. Inhibition of smpd-3 blocked EVs secretion and VSMCs calcification. Increased extracellular calcium induces smpd-3 expression and EVs production [17]. However, although high calcium levels have calcification, MGP expression can also be induced to delay vascular calcification. Nevertheless, as the calcium level increases, the content of MGP in MVs decreases and the protective effect is gradually weakened [24]. In addition, the identification of vascular calcification in patients with CD63 and CKD suggests that smpd-3 is a potential new therapeutic target for inhibiting EV production and vascular calcification [31].

3.3. Other mechanisms

Studies have shown that Tumor Necrosis Factor-α (TNF-α) can stimulate ECs to express bone morphogenetic protein-2 (BMP-2) and increase the formation of endothelial microparticles (EMPs) [33]. EMPs contain a large number of BMP-2 that induce osteogenic differentiation and calcification of VSMCs. ECs damage causes the release of EMPs with High levels of calcium and BMP-2, mediating inflammation and promoting vascular calcification [33]. Activated macrophages could release many soluble factors such as TNFα and also interleukin-1β (IL-1β) which are reported to enhance VSMCs osteogenic activity by increasing BMP2 production [34]. Reduced MGP and GRP levels could promote BMP2 osteogenic signaling to enhance VSMCs osteogenic differentiation and the release of calcifying competent EVs lacking MGP and GRP inhibitors. Interestingly, macrophages may directly impact on vascular calcification and capable of mineralization through the release of calcifying EVs enriched in S100A9 and annexin V, which contribute to accelerated microcalcification in chronic renal disease [35].

Studies have shown that MVs produced by aging ECs and MVs in plasma of the elderly can promote calcification of VSMCs [36]. Ca2+ and BMP2 carried by MVs in patients with chronic kidney disease and the elderly can promote osteogenic transformation of VSMCs [36]. ECs and VSMCs are linked to each other via EVs, affecting vascular function and AS formation. Under pathological conditions, changes in intercellular communication can cause a large number of myofibroblasts to secrete excessive collagen, cytokines and matrix metalloproteinases, leading to valve fibrosis and remodeling, promoting the production of osteoblast-like cells, ultimately resulting in vascular calcification [37].

4. Marking and therapeutic effects of EVs in vascular calcification

4.1. EVs can be used as biomarkers for diagnosis and prognosis

In various diseases, the number of EVs released by different cells is different from that of surface markers, and the contents of proteins, lipids, nucleic acids, and the like of the contents are also different. EVs are capable of transmitting specific molecular information to other cells, thereby affecting the function of the recipient cells. Interaction of EVs between cells can reflect disease states. EVs can be used as a non-invasive biomarker for the diagnosis and evaluation of prognosis, with disease-specificity, and play an important role in the early diagnosis of

cardiovascular and renal system diseases and cancer, etc. [38]. In vitro and in vivo studies have established miRNAs biomarkers for EVs that provide diseases with different circulating miRNA characteristics [14]. Muscle-specific miR-1 and miR-133a expression levels are elevated in serum of patients with acute coronary syndrome (ACS) and are associated with cardiac troponin T (cTnT) levels [39]. In cardiovascular disease, plasma EVs have been used as a source of nucleotide and protein markers. EV pIgR, cystatin C, and C5a have been identified as potential biomarkers for the diagnosis of ACS, and the mean EV concentration in the precipitated plasma EVs of all of these individual biomarkers was significantly associated with ACS in a total cohort of 471 suspected ACS patients. The association is significantly stronger in men than in women [40].

4.2. EVs can play a therapeutic role as a drug or drug carrier

EVs are small in size, easy to penetrate biofilm, and have low immunogenicity. The lipid bilayer membrane structure can protect the contents from degradation and ensure their biological activity [41]. Stem cell-derived EVs have regenerative potential and also show significant cardioprotective effects, with potential therapeutic effects on cardiac function after myocardial infarction [42]. Stem cell-derived EVs play a therapeutic role in various diseases such as vascular calcification by regulating the functions of vascular ECs and SMCs, anti-fibrosis, promoting cell and angiogenesis, and reducing apoptosis and other mechanisms [41]. The ability of these EVs to repair is to promote the expression of homeostatic and non-inflammatory phenotypes in diseased tissues by efficient delivery of miRNAs, providing a new therapeutic perspective for intercellular mediation of tissue damage and repair [43].

EVs are ideal drug carriers for direct transfer of drugs into their dual lipid membranes. It delivers the drug to the target cells and into the cells by membrane fusion or endocytosis. EVs are not toxic, can be tolerated by humans and have a long circulating half-life, but current research is mostly limited to animal experiments. Obtaining a small amount of EVs and low purity, its biodistribution and sustained biological effects, safety and effective dose require a lot of research and verification, and it is also the main problem of current research and application [44].

5. Prospect

EVs are considered a new way to treat cardiovascular disease. At present, the role of EVs in the treatment of cardiovascular diseases is confirmed only in animal experiments and at the cellular level. The clinical research data is relatively scarce, and the substances that play a role are still unclear. With the development of experimental techniques and molecular techniques, EVs treatment may become a clinically feasible treatment.

There are still many features of EVs that are not yet known. In humans, the specific components of EVs that play a role in vascular calcification have not been studied. At present, there is no method for treating vascular calcification. EVs are clinically important for the treatment of primary disease, which can fundamentally reduce the occurrence of vascular calcification and reduce SMC transdifferentiation and inflammatory factor activation.DDR-1 and smpd-3 are potential novel therapeutic targets for vascular calcification. Interventions in the formation and release of calcified EVs during vascular calcification and lowering serum phosphorus and calcium concentrations are effective in reducing cardiovascular disease and reducing mortality. We still need to understand the function of EVs and its role in the pathogenesis of vascular calcification, in order to find vascular calcification-specific EVs treatment in future research.

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Declaration of Competing Interest

None.

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