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Not all vascular smooth muscle cell exosomes calcify equally in chronic kidney disease

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Prevention of medial calcification in patients with chronic kidney disease requires the maintenance of vascular smooth muscle cell fitness. To preserve viability under chronic kidney disease–induced stress, vascular smooth muscle cells increase exosome formation and release, but the result is aggravated pathological calcification. Now Chen *et al.* report that microvesicles from calcifying vascular smooth muscle cells may propagate procalcifying signals to normal vascular smooth muscle cells. To help design effective strategies to impair procalcifying cell-to-cell communication, this commentary updates the current understanding of the main regulators of microvesicle/exosome biogenesis and secretion.

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Vascular calcification is the pathological deposition of calcium phosphate salts in the vasculature and is a prevalent and serious complication in patients with chronic kidney disease (CKD), where it associates positively with a disproportionately high risk of cardiovascular mortality. In the course of CKD, vascular calcification develops early, affecting 25% of patients at CKD stages 3 to 4 and increasing to 50%–80% in patients starting hemodialysis (reviewed in Paloian and Giachelli[1]).

In patients with CKD, calcification affects both the intimal and medial layers of the arterial wall. However, medial calcification, a process that resembles bone mineralization and is carried out by vascular smooth muscle cells (VSMCs), is more pronounced in CKD and is the exclusive form of vascular calcification in pediatric CKD (reviewed in Paloian and Giachelli[1]).

Important for treatment, despite similar clinical and biochemical risk factors, medial calcification progresses faster in hemodialysis patients with existing calcified lesions compared with patients with noncalcified CKD. In this issue of *Kidney International*, Chen and collaborators (2018) examine the ability of microvesicles generated by calcifying VSMCs cultured from arteries of rats with CKD to extend procalcifying signals to adjacent VSMCs cultured from normal rats.[2] The rationale for the study was the recognized key role of exosomes in cell-to-cell communication and the recent identification of a critical contribution of exosome biosynthesis and release by VSMCs to vascular calcification.[3] The results of the study by Chen *et al.* have revealed a greater complexity of mechanisms regulating intracellular control and compartmentalization of mineralization by VSMCs.

This commentary updates the current understanding and highlights the unanswered questions underlying microvesicle/exosome biogenesis and secretion from VSMCs. Ultimately, this knowledge will be essential to customize therapeutic strategies aimed at attenuating or delaying both the initiation of calcification and potentially the propagation of calcifying signals between VSMCs.

Figure 1 summarizes the mechanisms for pathological calcium deposition. First, VSMCs undergo a process of phenotypic transition that involves the loss of their contractile phenotype, required to maintain vascular tone, together with the upregulation of markers of osteochondrogenesis. Simultaneously, VSMCs release matrix vesicles that colocalize with elastin and collagen fibrils and form the nidus for mineralization.[1]

Recent characterization of the biogenesis of these calcifying matrix vesicles identified at least a subpopulation as exosomes, because the endosomal pathway and inward budding of the membrane of late endosomes or multivesicular bodies (MVB) participate in their formation,[3] as depicted in Figure 1. In addition, the role of

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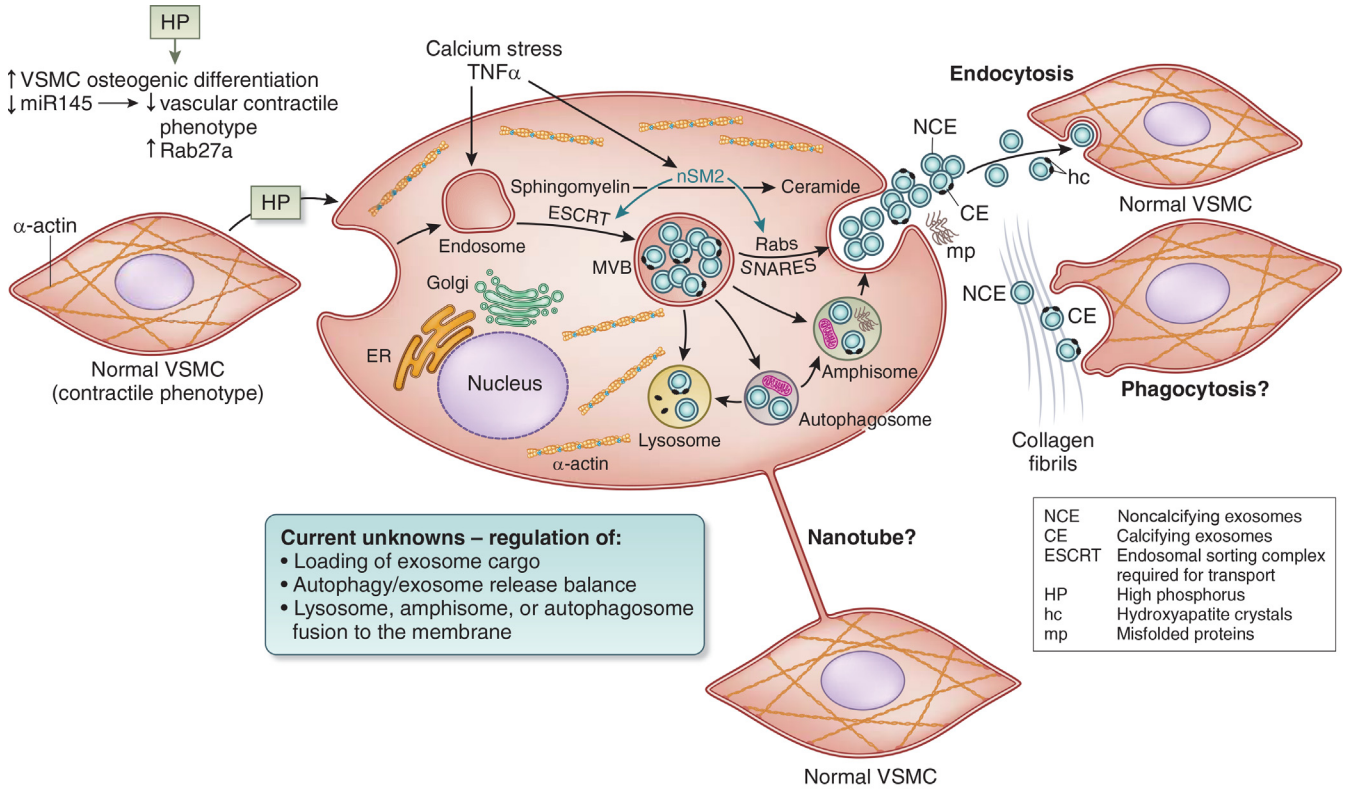

Figure 1 | Vascular smooth muscle cell (VSMC) exosome biogenesis and release. ER, endoplasmic reticulum; miR145, micro RNA 145; MVB, multivesicular bodies; nSM2, neutral sphingomyelinase 2; SNARES, Soluble NSF Attachment protein REceptor superfamily; TNF-α, tumor necrosis factor alpha.

sphingomyelin phosphodiesterase 3, also known as neutral sphingomyelinase 2 (nSM2), in the regulation of exosome release was shown. Furthermore, although elevated extracellular calcium and tumor necrosis factor α, common features in CKD, increased the expression of nSM2 and the secretion of calcifying exosomes, chemical inhibition of nSM2 prevented VSMC calcification.[3] These findings render the inhibition of nSM2 a novel strategy to attenuate calcification initiation. The severe bone mineralization defects in the nSM2 null mice strongly support the contribution of this mechanism to bone and vascular calcification.

Intriguingly, Chen and coworkers[2] demonstrate multiple procalcifying signals induced exclusively by the so called "cellular-derived exosome-like vesicles" obtained from collagenase digestion of VSMCs cultured from arteries of CKD rats upon their endocytosis by cultured VSMCs from normal rats exposed to high phosphorus. In contrast, there were no calcifying signals from exosome-like vesicles freely released into the incubation media by identical cells in culture. The calcifying signals from these matrix-trapped cellular-derived exosomes included increased intracellular calcium and oxidative stress in recipient VSMCs. However, exposure to high phosphate had no effect on the calcifying potency of the cellular exosome-like vesicles in increasing intracellular calcium and oxidative stress. They also showed that activation of transient calcium rises by these exosome-like vesicles was dependent on mitogen-activated protein kinase signaling; however, this was independent of the activation of oxidative stress and osteogenic gene expression. Thus, the authors show a potential contribution for intercellular signaling in propagating calcifying signals, but the mechanisms driving this calcification potential remain unclear. In addition, a critical unanswered question is how these procalcifying signals from matrix-trapped cellular-derived exosome-like vesicles reach a neighboring normal VSMC. One mechanism could be uptake from the extracellular matrix by phagocytotic processes. Alternatively, nanotubes, bridging CKD-derived VSMCs and normal VSMCs, could contribute, although the authors indicated that nanotube formation was not observed in these studies.

It is important to note that in the work by Chen *et al.* the vesicles were not fully characterized and therefore their composition as well as release and uptake pathways are difficult to determine. Both matrix-trapped and free-floating exosome-like vesicles were positive for CD63, but this is not necessarily indicative of an endosomal origin for both. Indeed, the main difference between these 2 vesicle populations was that the matrix-trapped population lacked fetuin-A, whereas the media-derived exosomes had a high fetuin content. These findings raise 2 important questions regarding the biogenesis of the 2 vesicle populations: (i) What is the origin of the fetuin in media-derived exosomes because VSMCs do not express fetuin? and (ii)

Why do matrix-derived exosomes, generated under identical stimulation, lack fetuin? Most likely the presence of serum and exosomes in the incubation media could account for the fetuin content, but it fails to explain why extracellular fetuin was not incorporated into matrix-derived vesicles. Alternatively, the media-derived exosomes could represent a heterogenous population of exosomes, with some coisolated from serum exosomes during ultracentrifugation. An important message from these controversial findings is that the existing enthusiasm for the use of exosome content as an early estimate of procalcifying activity awaits the development of better methods for exosome purification and characterization (reviewed in Hessvik and Llorente[4]). For example, high levels of extracellular phosphate were shown to be inefficient in inducing the generation of calcifying exosome-like vesicles, yet *in vivo* high phosphate is likely to be important because it induces systemic inflammation, favoring the induction of nSM2 expression and calcifying exosome release.[3] Furthermore, high phosphate causes reductions in micro RNA 145 in VSMCs.[5] Micro RNA 145 is[6] essential for the maintenance of VSMC contractile phenotype, and significantly, micro RNA 145 is a suppressor of the expression of Rab27a, an essential component of the endosomal sorting machinery required for exosome release.[7] Clearly, the degree of micro RNA 145 reduction induced by high serum phosphate and the resulting osteogenic differentiation of VSMCs could enhance Rab27a levels and exosome secretion in an nSM2-independent manner.

An additional consideration from the work by Chen *et al.*[2] is the colocalization with lysosome markers of the "cellular-derived" exosome-like vesicles endocytozed by normal VSMCs cultured in high phosphorus conditions. This points to a potential contribution of the balance between autophagy and exosome release according to physiological or pathological stimuli. High phosphate induction of autophagy was shown to attenuate the secretion of calcifying microvesicles and overall calcification. Also, under conditions that stimulate autophagy such as starvation, rapamycin treatment, or LC3 overexpression, MVBs are directed to the autophagy pathway, reducing exosome release. However, recent studies suggest that autophagy can also stimulate the release of MVB contents, including adenosine triphosphate,[8] which underscores the need for a better understanding of factors determining the balance between autophagy and exosome release, which may be regulated by the cellular metabolic state. Indeed, there is increasing evidence that both autophagy dysregulation and abnormal exosome secretion contribute to human disease (reviewed in Hessvik and Llorente[4]).

Similar to VSMCs, in chondrocytes incubated in osteogenic conditions, elevations in cytosolic calcium were followed by accumulation of annexins A2, A5, and A6 in calcifying matrix vesicles. Interestingly, a very recent publication has shown that the release of annexin 2 is stimulated by interferon gamma–induced autophagy. Moreover, inhibition of annexin 2 release was hampered under conditions that prevented fusion of autophagosomes with MVBs and of MVBs with the cell membrane,[9] such as Rab11, Rab8, and Rab27a knockdown. These recent results may also link autophagy with the release of calcifying matrix vesicles, although this hypothesis needs to be experimentally addressed.

There may be additional mechanisms linking exosome release and authophagy.[4] For example, cells lacking 2 autophagic proteins Atg12-Atg3 show hampered exosome release, probably because of alterations in late endosomal function, whereas alterations in the function of lysosomes, using the proton pump inhibitor Bafilomycin A1, cause a change in exosome cargos. Additionally, cellular stress conditions, such as a dysfunctional lysosomal pathway, endoplasmic reticulum stress by tunicamycin treatment, hypoxia, or irradiation, lead to an increase in exosome secretion. These increases in the release of exosomes may be a way of eliminating unwanted products accumulated in the cells. However, it is also likely that cells subjected to stress communicate with adjacent cells via the release of microvesicles. Thus, it is expected that VSMCs from CKD respond to different environmental and intracellular stressors, releasing exosomes with distinct cargos and procalcifying activities.

Understanding the complexity of these processes is the first step to optimize the control of exosome secretion and impair exosome-mediated cell-to-cell communication to safely minimize the risk of vascular calcification without an adverse impact on bone.

DISCLOSURE

All the authors declared no competing interests.

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Plasmacytoid dendritic cells: important players in human kidney allograft rejection

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Plasmacytoid dendritic cells are a unique dendritic cell subset that bridges innate and adaptive immune responses. They release high amounts of type I interferons in response to viral and bacterial infection. Plasmacytoid dendritic cells are thought to act as key players in renal allograft rejection, but the underlying mechanisms are unclear. Ruben *et al.* now demonstrate that granulocyte/macrophage colony-stimulating factor produced by renal epithelial cells is important to induce plasmacytoid dendritic cell maturation and indirect antigen presentation triggering allogeneic immune responses.

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Plasmacytoid dendritic cells (pDCs) are a rare immune cell type constituting less than 0.5% of human blood leukocytes. They derive their name from their light microscopic resemblance to both plasma cells and conventional dendritic cells, but they substantially differ from these immune cell types in terms of function and development. pDCs develop from a distinct hematopoietic precursor in the bone marrow under the influence of cytokines including fms-like tyrosine kinase 3 ligand, macrophage colony-stimulating factor, and thrombopoietin.[1] They traffic with the blood to lymph nodes, spleen, mucosal-associated lymphoid tissues, thymus, and liver.[1] Since the first reference to these cells 60 years ago, much has been learned about their ontogeny, phenotype, and function. The hallmark of pDC biology is the innate defense against infections. They sense the presence of viral single-stranded RNA through Toll-like receptor 7 and bacterial hypomethylated DNA through Toll-like receptor 9. These pathogen pattern recognition receptors engage the myeloid differentiation primary response protein 88-IRF7 pathway, resulting in the secretion of high amounts of type I interferons (IFN I).[1] These can stimulate cell-intrinsic antiviral defense mechanisms and augment the anti-infectious activities of NK, T, and B lymphocytes.[2]

The activation of T and B lymphocytes is the domain of conventional dendritic cells (DCs). Such DCs reside as sentinels in organs where they scan their environment for pathogens and sample antigenic material. Upon pathogen encounter, DCs undergo a maturation program and migrate into draining lymph nodes where they present the antigens to T lymphocytes, thereby inducing adaptive immunity. In the case of $CD8^+$ cytotoxic T lymphocytes, important for the defense against viral infections, such indirect antigen presentation is known as cross-presentation, a process well recognized to be regulated by IFN I produced from pDCs.[1] *In vitro* studies have shown that pDCs can present and cross-present antigen to $CD4^+$ and to $CD8^+$ T lymphocytes, respectively, but the *in vivo* relevance of indirect antigen presentation is unclear.

Conventional DCs in the kidney have been investigated intensively in the last several years and important regulatory roles have been uncovered in glomerulonephritis, pyelonephritis, acute ischemic injury, and other adverse conditions.[3] By contrast, the role of pDCs in renal disease is still poorly understood. These cells have been largely neglected by renal investigators, perhaps because viral infections and thus the need for type I interferon production are much less common in the kidney than in the liver, for example. Nevertheless, cells expressing the pDCs marker BDCA-2 have been detected in kidney sections from glomerulonephritis patients, but it remains unclear whether they produced IFN I, the hallmark function of pDCs. Experimental evidence exists for a role of pDCs as a pathogenic factor in lupus nephritis, a disease known to depend on IFN I.[1] An IFN I signature was detectable also in the blood of patients with antibody-mediated kidney transplant rejection,[4] and it coincided with an influx of pDCs into the renal tubulointerstitium and with the extent of fibrosis in this site. However, a causative role of pDCs in alloresponses has not yet been experimentally established in the context of kidney transplantation.

Kidney transplant rejection remains an important issue in clinical nephrology. Recent studies show that not only adaptive but also innate immune responses contribute to graft rejection. pDCs possess both innate and adaptive immune functions, i.e., their secretion of

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