Matrix vesicles induce calcification of recipient vascular smooth muscle cells through multiple signaling pathways

see commentary on page 298

Neal X. Chen[1], Kalisha D. O'Neill[1] and Sharon M. Moe[1,2]

[1]Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA; and [2]Roudebush Veterans Affairs Medical Center, Indianapolis, Indiana, USA

In patients with chronic kidney and end-stage renal diseases, the major risk factor for progression of arterial calcification is the presence of existing (baseline) calcification. Here, we tested whether calcification of arteries is extended from calcified vascular smooth muscle cells (VSMCs) to adjacent normal cells by matrix vesicle–induced alteration of cell signaling. Matrix vesicles isolated from VSMC of rats with chronic kidney disease were co-cultured with VSMCs from normal littermates. Endocytosis of vesicles by recipient cells was confirmed by confocal microscopy. The addition of cellular matrix vesicles with characteristics of exosomes and low fetuin-A content enhanced the calcification of recipient VSMC. Further, only cellular-derived matrix vesicles induced an increase in intracellular calcium ion concentration, NOX1 (NADPH oxidase) and the anti-oxidant superoxide dismutase-2 in recipient normal VSMC. The increase in intracellular calcium ion concentration was due to release from endoplasmic reticulum and partially attributed to the activation of both NOX1 and mitogen-activated protein kinase (MEK1 and Erk1/2) signaling, since inhibiting both pathways blocked the increase in intracellular calcium ion in recipient VSMC. In contrast, matrix vesicles isolated from the media had no effect on the intracellular calcium ion concentration or MEK1 signaling, and did not induce calcification. However, media matrix vesicles did increase Erk1/2, although not to the level of cellular matrix vesicles, and NOX1 expression. Blockade of NOX activity further inhibited the cellular matrix vesicle–induced accelerated calcification of recipient VSMC, suggesting a potential therapeutic role of such inhibition. Thus, addition of cellular-derived matrix vesicles from calcifying VSMC can accelerate calcification by inducing cell signaling changes and phenotypic alteration of recipient VSMC.

Kidney International (2018) **93,** 343–354; http://dx.doi.org/10.1016/j.kint.2017.07.019

KEYWORDS: calcium; chronic kidney disease; mineral metabolism; vascular calcification

Correspondence: *Sharon M. Moe, Division of Nephrology, Indiana University School of Medicine, 950 West Walnut Street, R2 202, Indianapolis, IN 46202, USA. E-mail: smoe@iu.edu*

Received 3 January 2017; revised 19 July 2017; accepted 20 July 2017; published online 9 October 2017

Vascular calcification is highly prevalent in chronic kidney disease (CKD) and is a major cause of morbidity and mortality.[1–3] The prevalence of calcification increases with worsening kidney disease[4]; by the time patients reach the dialysis stage, 70%–80% have significant coronary artery calcification.[5] On histology, medial calcification often begins to show itself as small areas within the medial layer. More-advanced lesions expand to become circumferential throughout the entire medial layer.[6] Risk factors for the presence of calcification in patients on dialysis include older age, diabetes, and disordered mineral metabolism, including hyperphosphatemia and hypercalcemia.[2] However, patients who have calcification at the start of dialysis have greater progression compared with those who do not have calcification, despite similar clinical and biochemical risk factors.[7] This difference suggests that expansion of existing calcification may occur through different mechanisms than does initiation of vascular calcification.

Studies from the past decade have led to increased understanding of the pathophysiology of vascular calcification. The VSMC must become synthetic with increased intracellular calcium[8,9] ($[Ca^{2+}]_i$) and downregulation of myocardin and alpha-smooth muscle actin,[10] followed by de-differentiation via upregulation of the "bone" (runt-related) transcription factor RUNX2.[11,12] These transformed, or de-differentiated, synthetic VSMCs initiate calcification by synthesizing small (50–200 nm) vesicles that initiate calcification on the extracellular matrix. In bone, these vesicles are called matrix vesicles (MVs), as they were identified as being an integral part of the conversion of hypertrophic chondrocytes in epiphyses of bones as they develop into mineralized bone[13,14]

Over the past decade, appreciation of the role of vesicles in cell–cell communication in nonmineralized tissues.has also increased.[15] Vesicles are heterogeneous and originate from the endosome or plasma membrane of cells. Although nomenclature and isolation techniques vary, vesicles can be released through outward budding of the plasma membrane (known as "shedding microvesicles") or inward budding of the endosomal membrane, resulting in the formation of multivesicular bodies.[16] We have previously characterized differences between

vesicles isolated from the media and the cells of calcifying bovine VSMCs: those from the media contain fetuin-A and do not readily mineralize, whereas those from cells do not contain fetuin-A and do mineralize.[3,17] Kapustin *et al.*[18] also compared vesicles from the media of calcifying human VSMC and found a similar proteomic profile to that of both cellular and media vesicles from osteoblasts.[18] Exosome production was increased by factors of clinical significance in CKD: increased extracellular calcium, tumor necrosis factor-α, and platelet-derived growth factor BB. They further identified these vesicles to be enriched with tetraspanins (CD9, CD63, and CD81), indicating origination from multivesicular bodies, and found such multivesicular bodies in calcified human arteries.[18] We and other groups have found that the origin and content of these MVs appears to be a central determinant of their mineralization potential.[3,19] This unique function, depending on content, is further supported by findings that vesicles isolated from atherosclerotic plaque (macrophage derived) and medial arterial calcification also differ in content.[20]

Multiple studies have demonstrated that vesicles can be taken up by recipient cells (these have been reviewed[21]). Given the pathologic appearance of vesicles in areas of vascular calcification *in vivo* and the role in calcification *in vitro*, we hypothesized that the transmission of vesicles from CKD cells to normal cells would facilitate calcification of the recipient cells and serve as a model of the extension or propagation of calcification observed in patients who have CKD. Given the parallel pathophysiology of both physiologic and pathologic calcification, we use the term matrix vesicles (MVs).

RESULTS

Cellular-derived, but not media-derived, MVs enhanced calcification of recipient VSMC

We compared 4 sources of MVs: cellular-derived MVs (from CKD VSMC incubated with either high-level phosphorus [calcifying] or normal-level phosphorus [control]) or media-derived MVs from calcifying or control CKD VSMC. These MVs were added to recipient normal rat VSMC as a co-culture and incubated with calcification media (high phosphorus) for 7 days. The results (Figure 1) revealed that both cellular sources of MVs from CKD VSMC (whether they were derived from donor VSMC incubated in normal-level or high-level phosphorus) induced calcification of the recipient VSMC incubated in high-phosphorus media. In contrast, MVs isolated from the media of cultured VSMC had no effect on calcification of recipient VSMC. These results suggest that the source of MVs, rather than the phosphorus conditions of the VSMC from which the MVs are derived, is what affects calcification. Figure 2a demonstrates that the MVs isolated from both sources of cellular VSMC contained annexin II, V, and VI, with higher expression in VSMCs that were incubated with additional phosphorus (calcifying). In contrast, the MVs isolated from the media had a lower level of annexins, and no differences were found whether they were from cells incubated in high-phosphorus media or not (Figure 2a and b). As

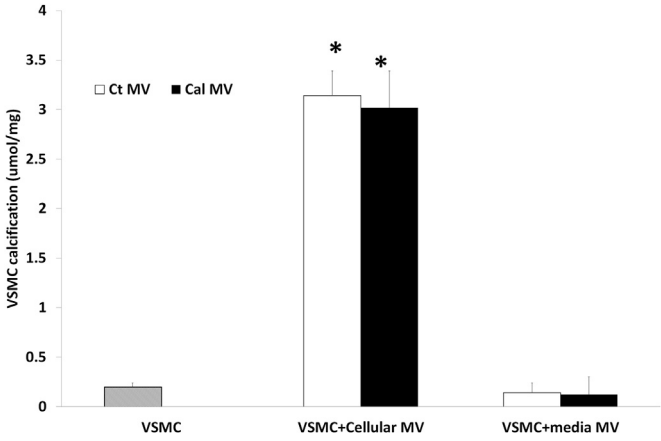

Figure 1 | Matrix vesicle (MV)—induced calcification of recipient VSMC. MVs were isolated from 4 sources of MVs: cellular-derived MVs (from chronic kidney disease [CKD] VSMC incubated with high phosphorus [5 mM β-glycerophosphate = [calcifying or Cal = black bars] or with normal phosphorus [no β-glycerophosphate] = control = Ct = white bars]) or media-derived MVs from calcifying or control CKD VSMC. Normal VSMC was incubated alone (hatched bar) or with cellular- or media-derived MV in the presence of β-glycerophosphate. Only the cellular origin of MV, regardless of whether they came from VSMC incubated in high or normal phosphorus, induced calcification of the recipient normal VSMC. Data are shown as mean ± SD ($n = 3$ MV sets from 3 CKD rats, with 3 cell cultures from each MV set, for a final n of 9). *$P < 0.05$, cellular MV versus VSMC alone or VSMC + media-derived MV.

we and others have found previously,[3,22] vesicles isolated from the cell media contained markedly increased fetuin-A. But again, whether the originating CKD VSMC cells were incubated with or without phosphorus made little difference (Figure 2a and b). Both cellular and media MVs from calcifying (high-phosphorous) conditions contain the exosomal tetraspanins CD63, CD81, and CD9, but cellular MVs are enriched with CD63, whereas media MVs are enriched with CD81 and CD9 (Figure 2a and b). These findings are consistent with those of Lotvall *et al.*,[23] who reported that although various types of extracellular vesicles contain many common exosome-enriched markers, such as tetraspanins, the relative proportions of these markers seem to vary in the different types. Despite these differences, examination by electron microscopy (Figure 2c) showed that both cellular and media MVs are membrane-bound vesicles of approximately 100 nm in diameter, consistent with the size of exosomes as described in the literature.[24] No nanotubes were identified by any imaging technique.[25]

Matrix vesicles are endocytosed by recipient VSMC

To determine if VSMC can uptake MVs, we labeled MVs with the membrane fluorescent dye PKH26 and examined uptake by confocal microscopy. The results revealed that MVs added to recipient VSMC were endocytosed by the VSMC (Figure 3a; *red*) and co-localized with Alexa 647–labeled dextran (Figure 3b; *blue*) but not transferrin (Figure 3c, *green*) after 24 hours. This finding indicates that once they are endocytosed, MVs become located in lysosome (Figure 3d