Actual. Osteol 2016; 12(2): 126-135.
Internet: http://www.osteologia.org.ar

ACTUALIZACIONES / *Reviews*

VASCULAR CALCIFICATION IN ATHEROSCLEROSIS: POTENTIAL ROLES OF MACROPHAGES AND NON-CODING MICRO-RNAs

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Abstract

Coronary heart disease, a leading cause of death in western societies, is caused by the presence of atherosclerotic plaques in the coronary arteries. Calcification is a frequent complication of atherosclerotic plaques, and often a contributing factor to their instability and rupture. Endothelial cells, smooth muscle cells and plaque macrophages, all contribute to the calcification process, which is reminiscent of that underlying bone formation. In particular, the role of macrophages in calcification has long been recognized, but whether or not distinct macrophage subsets –*v.g.*, M1 or inflammatory, and M2 or anti-inflammatory- have specific functions in osteogenic signaling within the context of plaque calcification remains poorly understood. Over the past few years, accumulated evidence has revealed novel roles of non-coding micro-RNAs (miRs) in atherorelevant functions of macrophages and in mechanisms linked to macrophage divergence into different subtypes. In this article we discuss some salient findings on potential roles of miRs in vascular calcification, with focus on those miRs that have also been associated to macrophage differentiation, and speculate on their potential relation to M1 and M2 macrophages in the context of calcification of atherosclerotic plaques.

Key words: atherosclerosis; vascular calcification; macrophage diversity; non-coding micro-RNAs

Resumen

CALCIFICACIÓN VASCULAR EN ATEROSCLEROSIS: POSIBLES FUNCIONES DE MACRÓFAGOS Y MICRO-ARNs NO CODIFICANTES

La enfermedad cardíaca coronaria, principal causa de muerte en occidente, es causada por la presencia de placas ateroscleróticas en las arterias coronarias. La presencia de depósitos de calcificación es una complicación frecuente de la placa, y puede contribuir a la inestabilidad y ruptura de la misma. El proceso de calcificación de la placa es similar al que ocurre en hueso, y contribuyen al mismo, mecanismos dependientes de células endote-

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liales, células musculares lisas y macrófagos, células que están presentes en todas las etapas de desarrollo de la placa aterosclerótica. El rol de los macrófagos en la calcificación de la placa se conoce desde hace tiempo, pero la contribución de los distintos tipos de macrófagos –por ejemplo, M1 o tipo inflamatorio, y M2 o tipo anti-inflamatorio- a mecanismos de señalización osteogénica en dicho contexto aún no se conoce. Recientemente varios trabajos experimentales han revelado la existencia de nuevos roles de micro-ARNs no codificantes (miRs) en varias funciones de los macrófagos que son de relevancia en el proceso aterogénico, como así también en mecanismos relacionados a la diferenciación de macrófagos en subtipos específicos. En este artículo discutimos algunos de los hallazgos más importantes sobre posibles nuevos roles de miRs en calcificación vascular, poniendo énfasis en aquellos miRs que han sido también asociados a la diferenciación de macrófagos, y especulamos acerca de su posible relación con macrófagos M1 y M2 en el contexto de la calcificación de la placa aterosclerótica.

Palabras clave: aterosclerosis; calcificación vascular; subtipos de macrófagos; micro-ARNs no codificantes.

1. Coronary artery disease and atherosclerosis.

Coronary heart disease (CHD) is the number one cause of death in the United States and other western societies.[1] The major cause of CHD is atherosclerosis, a chronic arterial disease that has a dominant inflammatory component.[2,3] The clinical expression of CHD includes chest pain –angina pectoris-, myocardial infarction and/or thromboembolic events, all of which are directly related to the molecular and cellular characteristics of the atherosclerotic plaque. For instance, plaques that cause critical arterial stenosis underlie ischemic manifestations, whereas rupture of unstable, vulnerable plaques usually derives in acute thromboembolic events.[4,5] One of the reasons why CHD has such a high impact on morbidity and mortality rates is because atherosclerosis is also the most frequent vascular complication of a number of metabolic disturbances, such as obesity, metabolic syndrome, diabetes and non-alcoholic fatty liver disease.[6]

Atherogenesis, the process that mediates plaque formation and growth, implies an intricate interaction between arterial resident cells, such as smooth muscle cells, endothelial cells and monocyte-derived macrophages, and a myriad of cytokines and inflammatory mediators that lodge in the plaque.[7] Desirable goals in the clinical management of atherosclerosis include minimizing plaque formation –in at risk individuals- or, in patients with established atherosclerosis, the reduction of plaque progression, improving the stability of potentially vulnerable plaques, and/or stimulating plaque regression. Calcification is a frequent complication of advanced atherosclerotic plaques, and depending on its extent and distribution within the lesion, it generally contributes to plaque instability and rupture.[8,9] Whereas existing therapies – *v.g.*, aggressive lipid lowering with statins- are somewhat successful in promoting plaque regression or preventing plaque development in individuals at risk, specific strategies to improve plaque stability by, for instance, reducing plaque calcification, are non-existent. In recent years, experimental evidence has revealed important functions of non-coding micro-RNAs (miRs) in different aspects of atherosclerosis, such as the regulation of components of the reverse cholesterol transport system,[10,11] modulation of macrophage differentiation (reviewed by us in[12]) and vascular calcification. In this article we discuss some of the salient findings regarding miRs and vascular calcification, with focus on those miRs that have also been linked to processes mediating macrophage

differentiation (see for instance [12]), and speculate on their potential relevance to the functions of M1 or inflammatory, and M2 or anti-inflammatory macrophage subsets in the context of calcification of atherosclerotic plaques.

2. Calcification in atherosclerotic plaques.

Calcification is a frequent complication of advanced atherosclerotic plaques, and depending on its extent –*i.e.*, micro or nodular calcification- and how it is distributed within the anatomy of the plaque –*i.e.*, cap shoulders, cap itself, necrotic area- it may represent a contributing factor to plaque instability and rupture. [4,8] The mechanisms that underlie vascular calcification are to a great extent reminiscent of those mediating bone formation, and calcified tissue found in atherosclerotic plaques is indeed indistinguishable, from a histological perspective, from bone. Bone related proteins such as Runt-related transcription factor-2 (Runx2), bone morphogenetic protein 2 (BMP2), osteoprotegerin, matrix gla protein, osteopontin, among others, are present in calcified plaques, and they fulfill most of the functions they would in a bone microenvironment.[13] For example, BMP2, which belongs to the family of bone morphogentic proteins (BMPs), a group of polypeptides in the transforming growth factor (TGF)-β superfamily, is a strong inducer of the differentiation of pre-osteoblasts into osteoblasts, mostly through activation of transcriptional factors required for bone formation.[14] One of these factors, Runx-2, is a master regulator of osteoblast differentiation and strongly activated downstream of BMP2 signaling. This mechanism is critical for BMP2 to induce endochondral bone formation in extra skeletal sites *in vivo.*[15] These processes also take place in the plaque environment, but the exact source of BMP2 is not fully defined and may include more than one cell type.

A key characteristic of vascular calcification in atherosclerosis is its predominantly intimal location, which distinguishes plaque calcification from that occurring in other chronic pathologies with an inflammatory vascular component –*i.e.*, chronic kidney disease- in which calcification is mostly found in the tunica media and is mainly subsequent to passive mineral deposition. At the cellular level plaque calcification involves an intricate interplay between endothelial cells, smooth muscle cells, and immune cells recruited to the vessel wall from circulation, most notably monocyte-derived macrophages.[16-18] Indeed, whereas vascular smooth muscle cells have been traditionally envisaged as the culprit of the osteogenic program behind vessel calcification, current evidence also supports key roles of endothelial cells and macrophages in this process.[16-18] A prominent mechanism by which macrophages induce calcification is by contributing to activation of the osteogenic program in vascular smooth muscle cells, specifically through the release of cytokines such as tumor necrosis factor-α (TNFα) or IL-6.[17,18] Macrophages can also de-differentiate into osteoclast-like cells which then contribute to the osteogenic process in the vessel by resorbing calcified mineral.[17] An additional mechanism may depend on the balance between accumulation of apoptotic macrophages and their clearance from the plaque through efferocytosis, a process key in the modulation of cellularity of early lesions and necrosis in advanced plaques.[19-21] Apoptotic bodies have an important nucleation capacity for the passive deposition of calcified mineral, and thus it is possible that, at least in the advanced lesion setting, the contribution of dying macrophages to the growth of the necrotic core through secondary necrosis may add to biomineralization of the matrix in the plaque (see Figure 1 for a summary of potential macrophage-related calcification mechanisms).

Despite the role of plaque macrophages in calcification being well-recognized, the

macrophage population in the plaque setting is diverse, and current knowledge on the specific roles of macrophage subsets in osteogenic signaling within the context of vascular calcification is poor. This is particularly important in the context of calcification of atherosclerotic plaques, as the M1 or inflammatory, or classically activated, and the M2 or anti-inflammatory, or alternatively activated macrophage types are dominant throughout all stages of plaque development.[12,22] The potential impact of macrophage diversity in mechanisms of plaque calcification, and for that matter, in most atherorelevant processes, is often ignored. Acknowledging this is of importance, as it may lead to definitions in the development of alternative therapeutic strategies in the clinical management of atherosclerosis and other vascular diseases with an inflammatory nature.

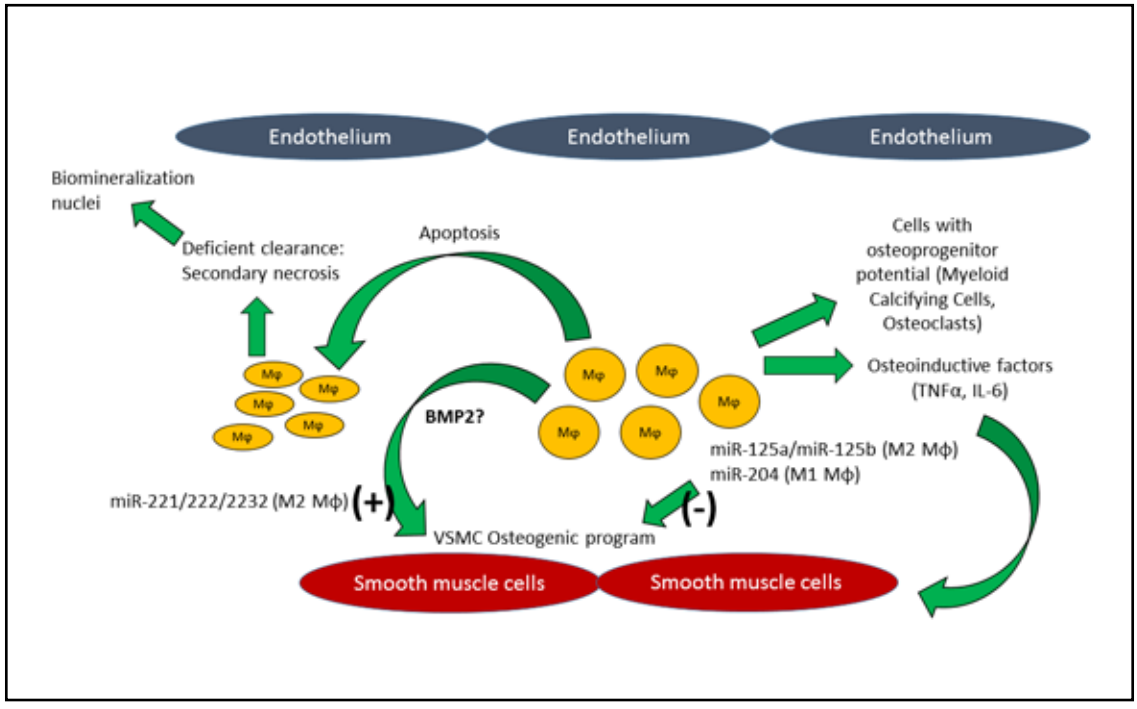

Figure 1. This figure summarizes some of the mechanisms by which macrophages (Mϕ) likely contribute to calcification of the atherosclerotic plaque. Under the influence of local cytokines and growth factors, macrophages can trans-differentiate into cells with osteoprogenitor potential (myeloid calcifying cells) or osteoclasts, which are key to the osteogenic process. Macrophages can also secrete osteoinductive factors (TNFα, IL-6) which may act on the vascular smooth muscle cell to induce an osteogenic program. Studies from the authors' laboratory suggest that bone morphogenetic protein-2 (BMP2) is constitutively produced by macrophages, which may represent an alternative pro-osteogenic mechanism in the plaque setting. In advanced atherosclerotic plaques, deficient clearance of apoptotic macrophages leads to secondary necrosis; necrotic debris can then serve as nuclei of biomineralization. Finally, some miRs that have been associated to regulation of osteogenic processes have also been identified in polarized M1 or M2 macrophages (indicated in parentheses; see text for details). The potential of these macrophage-derived miRs to stimulate (+) or inhibit (-) plaque calcification is yet to be demonstrated in animal models.

3. Macrophage diversity in the atherosclerotic plaque.

It is well documented that macrophage subsets exist in atherosclerotic plaques of humans and animal models, predominantly the M1 and the M2 types.[22-27] *In vitro*, the differentiation of naïve macrophages –usually bone marrow-derived- into the M1 or M2 types can be readily achieved by exposing the cells to, respectively, interferon-γ (IFN-γ) or interleukins 4 or 13 (IL4, IL13).[24,28] However, considering the myriad of stimuli to which macrophages are exposed in the plaque environment, this is a simplistic approach in a very controlled *in vitro* setting. This is why definitive evidence for the existence of fully divergent phenotypes in atherosclerotic plaques is still lacking. In this context, the growing number of studies applying deep transcriptomic analysis to plaque macrophages isolated from lesions by laser capture microdissection (LCM) is rapidly advancing our knowledge of the phenotypic signatures of plaque macrophages and how these compare to those observed *in vitro.*[29,30]

In atherosclerosis the relative abundance of M1 and M2 macrophages tends to increase as the disease progresses, although these two types co-exist throughout all stages of the disease and is likely that intermediate phenotypes are also present.[24,27] Whether the relative abundance of M1 and M2 macrophages in different stages of plaque development correlate with the extent and nature –*i.e.*, active *vs.* passive mineral deposition- of plaque calcification remains to be investigated. In this context, the application of RNA-seq analysis to interrogate the whole transcriptome of polarized macrophages should lead to identification of specific signatures of M1 and M2 macrophages, helping to shape and understand transcriptome pathways and potential molecular players with specific functions in vascular calcification. Although from a more canonical biochemical/cell biology approach, recent efforts started to shed light on potential functions of M1 or M2 macrophages in processes of relevance to vascular calcification. For example, evidence on potentially divergent roles of polarized macrophages in osteogenic signaling was recently provided by a study aimed at examining whether M1 and M2 macrophages differ in their ability to regulate the osteoblastic differentiation of mesenchymal stem cells *in vitro.*[31] Although a stronger osteogenic effect was observed for M2 macrophages compared to M1 cells, the authors attributed this effect to the pro-regenerative cytokines tumor growth factor-β and vascular endothelial growth factor produced by M2 macrophages,[31] with, surprisingly, no consideration given to macrophage-derived bone morphogenetic proteins. *In vitro* studies from our laboratory suggest that a mechanism exists in M1, but not M2 macrophages by which constitutive secretion of BMP2 drives an autocrine action that maintains the activation of a BMP2 receptor/SMAD1/5 signaling axis, which in turn accounts for constitutive expression of both BMP2 and Runx-2 (Dube and Vazquez, unpublished observations). Whereas the ability of macrophages from human and murine origin to produce BMP2 has been known for quite some time,[32] their ability to stimulate the osteogenic program in vascular smooth muscle cells has been attributed to the release of TNFα and IL6 by plaque macrophages.[18,31] Thus, on a speculative basis, constitutive BMP2 secretion by M1 macrophages may represent a novel osteogenic mechanism in the plaque setting. All these findings await validation in animal models of atherosclerosis.

4. Micro-RNAs, macrophage subsets and plaque calcification.

MicroRNAs (miRs) constitute a large family of small non-coding RNA molecules (~22 nucleotides) that can regulate gene expression at the post-transcriptional level by binding to the 3'-untranslated region (3'UTR) of target mRNAs, which results in repression of the

expression of that particular mRNA.[33] There is an impressive amount of evidence in support of functions of miRs in different molecular and cellular processes of atherosclerosis, with their roles in the regulation of components of the reverse cholesterol transport system in macrophages being one of the most studied thus far.[10,11,34,35] Recent studies also highlight observations on potential roles of miRs in vascular calcification, mostly focusing on their impact on the osteogenic capacity of smooth muscle cells. Below, we discuss some salient findings on miRs and vascular calcification and their potential relation to specific macrophage subsets in the context of calcification in the setting of atherosclerosis (and see Figure 1). The focus is on those miRs that have also been associated to processes that mediate macrophage differentiation into the M1 or M2 phenotypes. The rationale behind this selection lies on the fact that macrophages can secrete miRs packaged into exosomes –membranous nanovesicles-, which then act in a paracrine manner on neighbor cells[(36-38)]. We thus speculate that in the plaque setting macrophage-derived miRs with recognized roles in mechanisms of vascular smooth muscle cell calcification, may act in a paracrine manner on plaque smooth muscle cells to influence their osteogenic program.

Mir-125b has been known to be involved in osteoblastic differentiation, but its specific effect on vascular calcification has been unclear. Goetssch *et al.*[39] examined the effects of miR-125b in osteogenic transformation of vascular smooth muscle cells from human coronary artery, and found that expression of miR-125b was reduced when these cells were exposed to osteogenic medium.[39] Notably, expression of miR-125b was inversely correlated with mineralized matrix deposition, in agreement with the observation that suppressing endogenous miR-125b promoted osteogenic transdifferentiation. This effect occurred mostly at the expense of upregulation of the osteoblast transcription factor SP7 (osterix), a target of miR-125b. Of importance, calcified atherosclerotic plaques in apolipoprotein e knockout (apoeko) mice had reduced levels of miR-125b compared to non-calcified lesions, in line with a negative regulation of calcification processes by miR-125b.[39] In line with these findings, a miR-125b/Ets1 axis has recently been linked to regulation of transdifferentiation and calcification of vascular smooth muscle cells in high-phosphate medium.[40] Overexpression of miR-125b inhibited, while its downregulation promoted the phenotypic transition of smooth muscle cells and calcification, through a mechanism that involved miR-125b-dependent repression of Ets1.[40]

The close relative of miR-125b, miR-125a, has been associated to regulation of osteoclastogenesis. The differentiation of $CD14^+$ peripheral blood mononuclear cells induced by macrophage colony stimulating factor and receptor activator of nuclear factor kappa B ligand (RANKL), was shown to be markedly reduced or promoted by, respectively, overexpression or downregulation of miR-125a, through a mechanism that involved miR-126a-mediated targeting of TNF receptor-associated factor 6 (TRAF6), which acts as a signaling element in the RANKL/RANK/NFATc1 pathway.[41]

Interestingly, microarray analysis of miR profiles in bone marrow-derived macrophages from Balb/c mice showed that expression of miR-125b-5p is significantly higher in M2 compared to M1 macrophages.[42] In addition, studies in bone marrow-derived macrophages from C57BL/6 mice showed that stimulation of Toll-like receptors (TLR) 2 and 4 favors expression of miR-125a-5p, whereas miR-125b-5p, which has the same seed sequence as miR-125a-5p, was downregulated.[43] This and the studies above illustrate the fact that sequence context outside the seed region can also affect miR expression and their binding to targets. Macrophages overexpressing miR-125a-5p

differentiate readily to the M2 phenotype, whereas knockdown of miR-125a-5p promotes M1 differentiation.[43] In the plaque setting macrophages are exposed to a variety of stimuli that induce endoplasmic reticulum (ER) stress, and both TLR2 and TLR4 are key within the signaling associated to the ER stress response in macrophages.[44] It is tempting to speculate that under regressive plaque conditions, where M2 macrophage abundance increases, release of upregulated miR125a to the environment may favor calcification regression by interfering with osteoclastogenic mechanisms.

Eigsti *et al*.[45] who found that during the transition monocyte-to-macrophage, IL4 –an M2 inducer– promoted the expression of miR-222. Recent miR-microarray analysis in smooth muscle cells revealed that after an extended period of time in culture the expression of miR-222 and its close relative miR-221 are downregulated, and this was positively correlated with lack of an osteogenic phenotype.[46] However, transfection of smooth muscle cells with miR-221/miR-222 mimics resulted in increased calcium deposition, and this was associated to changes in activity of the ectonucleotide phosphodiesterase 1 (Enpp1) and Pit-1 expression. The conclusion was made that these miRs modulate smooth muscle cell-mediated calcification by promoting cellular inorganic phosphate and pyrophosphate levels.[46]

MiR-223, a critical factor in osteoclastogenesis, is markedly upregulated in vascular smooth muscle cells exposed to high phosphate environment and in aortic plaques from apoeko mice with vascular calcification.[47] Interestingly, miR-223 is highly specific of IL4-induced M2 macrophages, and has indeed been shown to be transferred by microvesicles into naïve macrophages to promote M2-like differentiation.[37] It remains to be determined if such paracrine mechanism also operates in the plaque setting and whether macrophage-derived miR-223 can be transferred to vascular smooth muscle cells to induce the osteogenic program.

Expression of miR-204 has been found to be negatively correlated with osteoblastic differentiation of mouse aortic vascular smooth muscle cells.[48] Whereas high phosphate environment suppressed miR-204 expression, overexpression of miR-204 decreased Runx-2 levels and alleviated high-phosphate induced calcification of smooth muscle cells. *In vivo*, administration of miR-204 mimetics to mice markedly attenuated vitamin D_3-induced vascular calcification.[48] Interestingly, miR-204 levels are increased in M1 macrophages –induced with lipopolysaccharide plus IFNγ– compared to M2 cells.[42] Thus, M1-dependent secretion of miR-204 may attenuate osteoblastic differentiation of precursor cells in the plaque by repressing Runx2-dependent osteogenic signaling in plaque smooth muscle cells.

5. Concluding remarks.

Calcification of atherosclerotic plaques adds to the complexity of the mechanisms mediating plaque growth and instability. Much progress has been made in understanding molecular and cellular mechanisms that underlie plaque calcification, which to a great extent recapitulate the processes that mediate bone formation. Macrophages have long been recognized as key cellular components of vascular calcification, but their role has been for the most part attributed to the release of cytokines and/or their capacity to contribute as osteoclast-like cells to the osteogenic process. The diversity of the macrophage population in the plaque setting obliges to explore the potentially divergent roles of polarized macrophages in osteogenic signaling. As discussed above, some recent studies have begun to provide evidence to help us understand such conundrum.

Interestingly, a number of miRs that have been associated to specific macrophage subsets have also emerged as potential

regulators of distinctive processes in the osteogenic mechanisms mediating plaque calcification. Whether these miRs can be secreted by distinct macrophage subsets and act in a paracrine manner on plaque smooth muscle cells to influence specific aspects of their osteogenic program is an attractive mechanism that merits investigation. Additional studies on the transcriptome of plaque macrophages are also needed to further our knowledge of subset-specific miRs and their potential link to calcification relevant events. *In vivo* validation of such miRs may lead to identification of potential therapeutic targets to manipulate macrophage and/or smooth muscle cell osteogenic components to prevent or ameliorate plaque calcification.

Acknowledgments
This work was supported by NIH grant R01HL111877 (G.V.) and University of Toledo College of Medicine and Life Sciences.

Conflict of interests The authors declare no conflicts interest.

(Recibido: junio 2016.
Aceptado: julio 2016)

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