EUROPEAN SOCIETY OF CARDIOLOGY®

Cardiovascular Research (2013) **100**, 472–480
doi:10.1093/cvr/cvt206

The galectin-3/RAGE dyad modulates vascular osteogenesis in atherosclerosis

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Received 22 February 2013; revised 16 August 2013; accepted 20 August 2013; online publish-ahead-of-print 23 August 2013

Time for primary review: 27 days

Aims Vascular calcification correlates with inflammation and plaque instability in a dual manner, depending on the spotty/granular (micro) or sheet-like/lamellated (macro) pattern of calcification. Modified lipoproteins trigger both inflammation and calcification via receptors for advanced lipoxidation/glycation endproducts (ALEs/AGEs). This study compared the roles of galectin-3 and receptor for AGEs (RAGE), two ALEs/AGEs-receptors with diverging effects on inflammation and bone metabolism, in the process of vascular calcification.

Methods and results We evaluated galectin-3 and RAGE expression/localization in 62 human carotid plaques and its relation to calcification pattern, plaque phenotype, and markers of inflammation and vascular osteogenesis; and the effect of galectin-3 ablation and/or exposure to an ALE/AGE on vascular smooth muscle cell (VSMC) osteogenic differentiation. While RAGE co-localized with inflammatory cells in unstable regions with microcalcification, galectin-3 was expressed also by VSMCs, especially in macrocalcified areas, where it co-localized with alkaline phosphatase. Expression of galectin-3 and osteogenic markers was higher in macrocalcified plaques, whereas the opposite occurred for RAGE and inflammatory markers. Galectin-3-deficient VSMCs exhibited defective osteogenic differentiation, as shown by altered expression of osteogenic transcription factors and proteins, blunted activation of pro-osteoblastogenic Wnt/β-catenin signalling and proliferation, enhanced apoptosis, and disorganized mineralization. These abnormalities were associated with RAGE up-regulation, but were only in part prevented by RAGE silencing, and were partially mimicked or exacerbated by treatment with an AGE/ALE.

Conclusion These data indicate a novel molecular mechanism by which galectin-3 and RAGE modulate in divergent ways, not only inflammation, but also vascular osteogenesis, by modulating Wnt/β-catenin signalling, and independently of ALEs/AGEs.

Keywords Vascular calcification • Inflammation • Galectin-3 • RAGE • Wnt/β-catenin

Introduction

Vascular inflammation and calcification are strictly related phenomena occurring within the vessel wall during atherogenesis and contributing to plaque instability. The mechanisms by which inflammation favours lesion progression include induction of apoptosis and necrosis of macrophages and vascular smooth muscle cells (VSMCs) and activation of matrix degrading processes leading to plaque rupture and intra-plaque haemorrhage.[1] In addition, inflammation may trigger vascular calcification via several mechanisms, including reduction of calcification inhibitors, formation of tissue nucleational complexes, and induction of VSMC osteogenic differentiation.[2] However, vascular calcification seems to correlate with plaque instability in a dual manner, depending on the type and extent of calcification.[3] In fact, in response to inflammatory stimuli, VSMCs produce matrix vesicles, where calcium precipitates to form calcification nuclei. Extension of the inflammatory process leads to formation of additional spotty or granular calcium deposits (microcalcification), a vicious cycle favouring further tissue damage and leading to plaque instability. On occasion, the process of calcification may switch towards formation of large plates of sheet-like, lamellated, homogeneous calcium

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deposition (macrocalcification), which is accompanied by little or no inflammation, thus maintaining plaque stability.

That micro and macrocalcification differ for rate of lesion progression and severity of clinical outcomes has been demonstrated by an intravascular ultrasound examination. Microcalcification predominated in patients with acute myocardial infarction, whereas macrocalcification prevailed in subjects with stable angina pectoris.[4] A recent report has confirmed that spotty calcification is a marker of accelerated progression of coronary atherosclerosis.[5] However, the molecular mechanisms underlying the switch from micro to macrocalcification, i.e. from spotty/granular to sheet-like/lamellated calcification, and the dual mode by which calcification correlates with both inflammation and plaque instability have not been investigated.

Vascular inflammation results from multiple local and systemic mechanisms, including modification of lipoproteins, particularly by lipoxidation and non-enzymatic glycation leading to formation of a heterogeneous group of compounds collectively referred to as advanced lipoxidation endproducts (ALEs)[6] and advanced glycation endproducts (AGEs),[7] respectively, which share biological effects[6,7] and immunogenic properties.[8] ALE/AGE-specific epitopes found in modified lipoproteins represent a prominent set of damage-associated molecular patterns, which are recognized by pattern recognition receptors (PRRs) of innate immunity. PRRs include the classical ALE/AGE receptors galectin-3 and receptor for AGEs (RAGE).

While RAGE has been conclusively demonstrated to favour the development of atherosclerotic lesions by mediating the pro-inflammatory effects of AGEs/ALEs,[9] the role of galectin-3 in atherogenesis is controversial. However, the fact that galectin-3 mediates internalization and degradation of these by-products[10] suggests an anti-inflammatory effect, consistent with our finding that galectin-3-deficient animals show accelerated atherogenesis on a high-fat diet.[11] Moreover, a role of RAGE in mediating vascular calcification induced by its ligands AGEs/ALEs[12] and calgranulins[13] has been demonstrated both *in vivo* and *in vitro*, whereas it is unknown whether galectin-3 participates in this process. Interestingly, galectin-3 and RAGE have been shown to play a role in bone formation and resorption, respectively, thus modulating skeletal tissue homoeostasis.[14,15]

This study compared the roles of galectin-3 and RAGE, two ALE/AGE-binding proteins with opposite effects on both vascular inflammation and bone metabolism, in the process of plaque mineralization leading to formation of micro (spotty/granular) or macro (sheet-like/lamellated) calcification. Results showed that the galectin-3/RAGE dyad participates in vascular osteogenesis by modulating activation of pro-osteoblastogenic Wnt/β-catenin signalling, with the two receptors showing different plaque expression patterns and exerting diverging effects on VSMC osteogenic differentiation.

2. Methods

See also Supplementary material online, Detailed Methods.

2.1 *In vivo/ex vivo* studies

In these studies, we assessed the expression and localization of galectin-3 and RAGE in human carotid plaques and their relation with plaque phenotype (stable or unstable), type of calcification (spotty/granular or sheet-like/lamellated), and markers of inflammation, fibrosis, and vascular osteogenesis. Carotid plaques were obtained from 62 consecutive patients undergoing carotid endoarteriectomy (CEA) for symptomatic or asymptomatic, haemodynamically significant carotid stenosis (>70%). This investigation conforms with the principles outlined in the Declaration of Helsinki and an approval was granted by the Ethics Committee of the Sant'Andrea Hospital.

Carotid sections were analysed semi-quantitatively (scores: 0 = absent, 1 = mild; 2 = moderate; 3 = heavy) for evaluating parameters of plaque instability, inflammation, fibrosis, and calcification (Supplementary material online, *Table S1*). Plaques were characterized according to well-defined histological criteria, which are associated with clinical instability.[16] In detail, established markers of plaque instability and rupture, such as haemorrhage and thrombus,[17] and neovascularization of the intima[18] were graded. Then, an overall instability index for each plaque was calculated by adding the scores for haemorrhage, thrombosis, and intimal neovascularization. Opposite determinants of plaque vulnerability, such as inflammation and fibrosis, were graded separately. Plaques were also scored for the presence of dystrophic micro and macrocalcifications. Spotty/granular calcifications were identified as aggregates of granular and heterogeneous deposits of basophilic crystalline material[17] with an average size of each granule of $0.0032 \pm 0.00021\ mm^2$, corresponding to a circle of ~20 μm diameter. This size is congruent with the definition of microcalcification found in the literature.[19] Conversely, macrocalcifications were recognized as large plates of lamellated, homogeneous calcium deposition, with a sheet-like appearance[17] and an average size of $1.64 \pm 1.19\ mm^2$. Plaques were then classified as microcalcified, macrocalcified or mixed, based on the presence or absence of a predominant calcification pattern. Finally, a total calcification score was obtained by adding the scores for microcalcification and macrocalcification. The presence of bone within the atherosclerotic plaque was scored positive when any focus of bone matrix, and bone cells were both present.

The expression of RAGE and galectin-3, the AGE/ALE N^{ε}-carboxymethyllysine (CML),[20] the RAGE ligand S100A12,[21] and the marker of osteogenic differentiation alkaline phosphatase liver/bone/kidney (tissue non-specific, ALPL) were evaluated immunohistochemically.[11,22] Cell distribution was evaluated by both immunoperoxidase on sequential sections and dual label immunofluorescence. Activity of tartrate-resistant acid phosphatase (TRAP) was assessed using the TRAP acid phosphatase leucocyte kit (Sigma, St Louis, MO, USA). The gene expression of the following molecular markers was assessed by real-time (RT)–PCR[11,22] only in plaques with a prevalent pattern of calcification (i.e. microcalcification or macrocalcifications/ossification): galectin-3, RAGE, S100A12, collagen I, the VSMC marker α-smooth muscle cell actin (α-SMA), the inflammation markers interferon (INF)-γ and tumour necrosis factor (TNF)-α, the osteogenic markers ALPL and bone gamma-carboxyglutamate (gla) protein (BGLAP), the osteogenic transcription factors Runt-related transcription factor 2 (Runx2) and bone morphogenetic protein (BMP)-2, and the non-specific osteoclast marker TRAP. Results were expressed as the ratio of the target gene transcript to the reference gene transcript (β-actin).

2.2 *In vitro* studies

In these studies, we evaluated the effect of galectin-3 ablation and/or exposure to an ALE/AGE on VSMC osteogenic differentiation at 1, 2, and 3 weeks. VSMCs were isolated from aortas obtained from adult (aged 6 weeks) female galectin-3-deficient mice (*Lgals3*$^{-/-}$),[11,22] and the corresponding C57BL/6J wild-type (WT) mice. Then, RAGE was silenced in WT and *Lgals3*$^{-/-}$ cells by transfecting them with silenced RAGE (siRAGE) expression vector and negative control or scramble according to the manufacturer's protocol. Cells were grown in DMEM plus 10% foetal calf serum, supplemented with either 10 mM β-glycerophosphate, 4 mM $CaCl_2$, and 50 μg/mL ascorbic acid (osteogenic medium) or vehicle (normal medium),[23] with or without addition of 100 μg/mL CML-modified mouse serum albumin (MSA), prepared as previously reported,[24] unmodified MSA or vehicle. Then, monolayers were processed for molecular and morphological analysis of osteogenic differentiation.

Nuclear translocation of β-catenin was evaluated by western blot, whereas the mRNA expression of osteogenic genes and target genes of the Wnt/β-catenin pathway were assessed by RT–PCR. VSMC apoptosis

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and proliferation/viability were also assessed. Total calcification area and number and size of calcification nodules were assessed morphometrically in monolayers stained with Von Kossa. To account for calcium deposits not detectable at light microscopy analysis, total calcium content of monolayers was measured using a colorimetric assay (Quantichrom Calcium Assay Kit, BioAssay Systems, Hayward, CA, USA). The analysis of gene expression was performed by RT–PCR.

2.3 Statistical analysis

Results are expressed as means ± SD, median (inter-quartile range), or number of cases and percentage.

Linear regression analysis was performed to assess correlation between calcification scores and plaque instability and inflammation indexes or galectin-3 and RAGE expression/distribution.

The Mann–Whitney test, for continuous variables, and the Pearson χ^2 test, for categorical variables, were applied to compare clinical findings and data from micro and macrocalcified plaques. In the cell culture studies, differences between genotypes and treatments were assessed using Student's t test or the one-way ANOVA followed by the Student–Newman–Keuls test, for parametric data, and the Mann–Whitney test or the Kruskal–Wallis test, for non-parametric data.

A P-value < 0.05 was considered significant. All statistical tests were performed on raw data, using the SPSS 10.0 statistical software.

3. Results

3.1 *In vivo/ex vivo* studies

3.1.1 Patients' clinical characteristics

See the Online-only supplementary material and Supplementary material online, *Table S2*.

3.1.2 Histological and molecular plaques characterization

Histological analysis revealed calcification in virtually all the plaques analysed. Most of them showed a mixed pattern of calcification (26/62, 41.94%), whereas predominance of either microcalcification (specimens scoring 3 for micro and 0–1 for macro) or macrocalcification (specimens scoring 0–1 for micro and 3 for macro) was observed in 18/62 (29.03%) of the plaques. Moreover, 11/18 (61.1%) of macrocalcified plaques (17.2% of all plaques) showed formation of mature lamellar bone, in which all bone cell types and bone marrow could be identified (Supplementary material online, *Figure S1*).

Figure 1 shows representative images of plaques with a prevalent spotty pattern of calcification (microcalcification), associated with inflammation and instability features, and a plaque with homogenous, lamellated and extended mineralization (macrocalcification), associated with fibrosis and a more stable phenotype. Linear regression analysis carried out in all samples showed a weak, though significant, correlation between the total calcification score and the overall instability index ($R^2 = 0.162$, $P < 0.05$); however, separate analysis showed a stronger positive correlation of the instability index with the score for microcalcification ($R^2 = 0.711$, $P < 0.001$), and a negative one with the macrocalcification score ($R^2 = 0.352$ $P < 0.001$). Likewise, weak correlation, albeit statistically significant, was found between total calcification and inflammation scores ($R^2 = 0.127$, $P < 0.05$), with a clear positive ($R^2 = 0.602$ $P < 0.001$) and negative ($R^2 = 0.314$, $P < 0.001$) trend for micro and macrocalcification, respectively (Supplementary material online, *Figure S2*).

RT–PCR analysis of plaques (*Figure 2*) revealed higher expression of INF-γ, TNF-α, and RAGE and slightly though statistically significantly lower levels of galectin-3 in microcalcified plaques. Also expression of S100A12 was higher in microcalcified plaques (not shown). Moreover, expression of collagen I, ALPL, and BGLAP were higher in macrocalcified plaques, whereas expression of TRAP was higher in microcalcified plaques. Slightly or no significant differences, respectively, were observed in the expression of Runx2 and BMP-2. Immunofluorescence analysis confirmed the expression level of galectin-3 and RAGE in microcalcified, macrocalcified, and mixed plaques (Supplementary material online, *Figure S3*). Interestingly, symptoms of stroke or transient ischaemic attack (TIA) were present in 3/18 subjects (16.7%) with prevailing sheet-like/lamellated calcification (macrocalcified plaque) and in 12/18 patients (66.7%) with prevailing spotty/granular calcification (microcalcified plaque) ($P = 0.002$). No significant difference in the above parameters was found between plaques from non-diabetic and diabetic subjects as well as from patients with and without chronic kidney disease (CKD). However, this finding could be due to a selection effect related to the cross-sectional design of the study, which included subjects with a cerebrovascular event from groups of individuals at different risk rate for that event as well as death. There was only a non-significant trend (75 vs. 50%) of diabetic patients to show mature bone formation in macrocalcified plaques, which is consistent with a previous report[17] and might be explained by the finding that diabetes increases the circulating levels of osteo-progenitors cells.[25]

3.1.3 Galectin-3 and RAGE distribution in tissue plaque

Galectin-3 was expressed by lipid-laden cells and infiltrating inflammatory cells, but also by VSMCs in fibrous areas (*Figure 3A and B*) and, especially, in regions adjacent to sheet-like/lamellated calcifications (*Figure 3C*). In unstable, inflamed plaque regions, VSMCs expressed little, if any, galectin-3, whereas in fibro-calcific, non-inflamed plaque areas where macrophages are rare and VSMCs predominate, most VSMCs expressed galectin-3 (Supplementary material online, *Figure S4*). In macrocalcified plaques, galectin-3 showed a pattern of positivity similar to that of the VSMC marker α-SMA and the marker of osteogenic differentiation ALPL (*Figure 3C*). Interestingly, also the subcellular distribution pattern of galectin-3 differed between inflammatory cells and VSMCs, since it was predominantly cytoplasmic and nuclear, respectively (Supplementary material online, *Figure S5*).

Instead, RAGE positivity was higher in plaques with prevalent spotty/granular calcification and high instability index (2.61 ± 0.70 vs. 1.27 ± 0.46; $P < 0.001$), and was typically observed in regions rich in foam cells and/or inflammatory infiltrate co-localizing with monocytes–macrophages (*Figure 3D* and Supplementary material online, *Figure S6*). The intensity and distribution of CML staining recapitulated that of RAGE and also S100A12 positivity was confined to microcalcified, unstable plaques (Supplementary material online, *Figure S7*).

Intense enzymatic TRAP activity was shown by microcalcified, inflamed and unstable plaques, with clusters of inflammatory cells displaying strong positivity. In contrast, TRAP activity was low in macrocalcified plaques, being mainly confined to cells with an osteoclastic phenotype in ossified regions (Supplementary material online, *Figure S8*).

Again, no significant effect of diabetes or CKD on these parameters was detected.

3.2 *In vitro* studies

3.2.1 Molecular markers

Osteogenic differentiation induced by osteogenic medium in *Lgals3*$^{-/-}$- and WT-VSMCs was evaluated at 1, 2, and 3 weeks. RT–PCR demonstrated that the osteogenic transcription factor Runx2 and the osteogenic marker ALPL increased over time in WT-VSMCs, whereas

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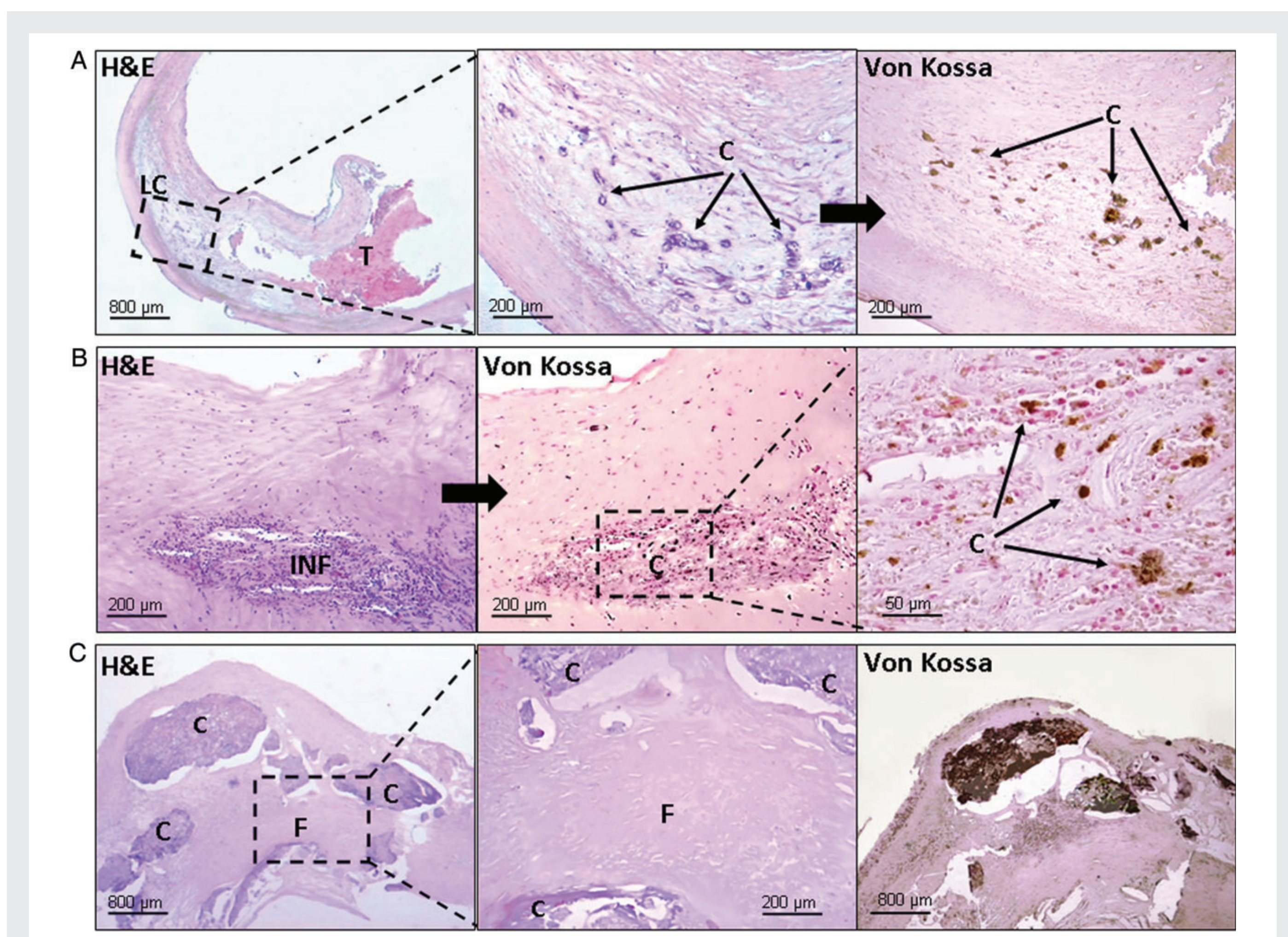

Figure 1 Haematoxylin and eosin (H&E) and Von Kossa staining of representative serial sections of unstable (*A* and *B*, instability index 6 and 5, respectively) and stable (*C*, instability index 0) plaques. Atherosclerotic plaques with large lipid cores (LC), thrombosis (T) and inflammatory infiltrate (INF) show a spotty/granular pattern of calcification (*C*), as identified by aggregates of basophilic crystalline material in H&E-stained sections or brown-black granules in Von Kossa-stained sections. Plaques with no features of instability or inflammatory cells infiltration show extensive fibrosis (*F*) and large plates of sheet-like/lamellated calcification (*C*).

the VSMC marker α-SMA decreased, when compared with cells grown in normal medium. *Figure 4* shows 3-week values. Interestingly, galectin-3 expression was up-regulated by the osteogenic stimulus in a fashion similar to ALPL. RAGE and TRAP were also up-regulated, albeit to a lesser extent. However, comparing the absolute values, galectin-3 expression was 23 times higher than that of RAGE (not shown). More importantly, galectin-3 deficiency hampered the ability of VSMCs to adequately differentiate towards an osteogenic phenotype, as demonstrated by the blunted expression of Runx2 and ALPL, and the attenuated loss of α-SMA. Furthermore, galectin-3 ablation was associated with increased expression of RAGE in normal medium, as previously shown by our group in mesangial cells and aortic and renal tissues from galectin-3-deficient mice,[11,22] and a marked up-regulation of both RAGE and TRAP in osteogenic medium, when compared with WT-VSMCs. To assess whether the abnormal osteogenic differentiation of *Lgals3*$^{-/-}$-VSMCs was due to galectin-3 deficiency or the associated RAGE overexpression, RAGE was silenced in these cells. However, only Runx2 returned to values not significantly different from those of WT cells, whereas ALPL expression was unchanged, and TRAP and α-SMA mRNA levels were closer to those of *Lgals3*$^{-/-}$ than to those of WT cells. This supports the concept that the effect of galectin-3 deficiency was not attributable to, though it was accentuated by, RAGE up-regulation. To get insight into the mechanism by which galectin-3 affects osteogenic differentiation of VSMCs, we then investigated the Wnt/β-catenin signalling. The rationale for these experiments is based on the well-documented role of Wnt/β-catenin in osteoblastogenesis[26] as well as on data showing that galectin-3 is a key regulator of this pathway through its ability to interact with and translocate β-catenin into the nucleus,[27] whereas RAGE inhibits osteoblast proliferation through suppression of β-catenin/Wnt signalling.[28] Results showed that, in WT- but not *Lgals3*$^{-/-}$-VSMCs, nuclear translocation of β-catenin was induced by 24-h exposure to osteogenic medium and maintained at 1 week. A parallel trend was observed in the expression of the β-catenin target genes cyclin D1 and axin2 (the latter playing an important role in the regulation of β-catenin stability), thus supporting the concept that Wnt/β-catenin signalling was activated in WT but not *Lgals3*$^{-/-}$ cells (*Figure 5*). Exposure to osteogenic medium induced apoptosis in *Lgals3*$^{-/-}$, but not WT cells, whereas it increased proliferation/viability in WT-, but not *Lgals3*$^{-/-}$-VSMCs (Supplementary material online, *Figure S9*).

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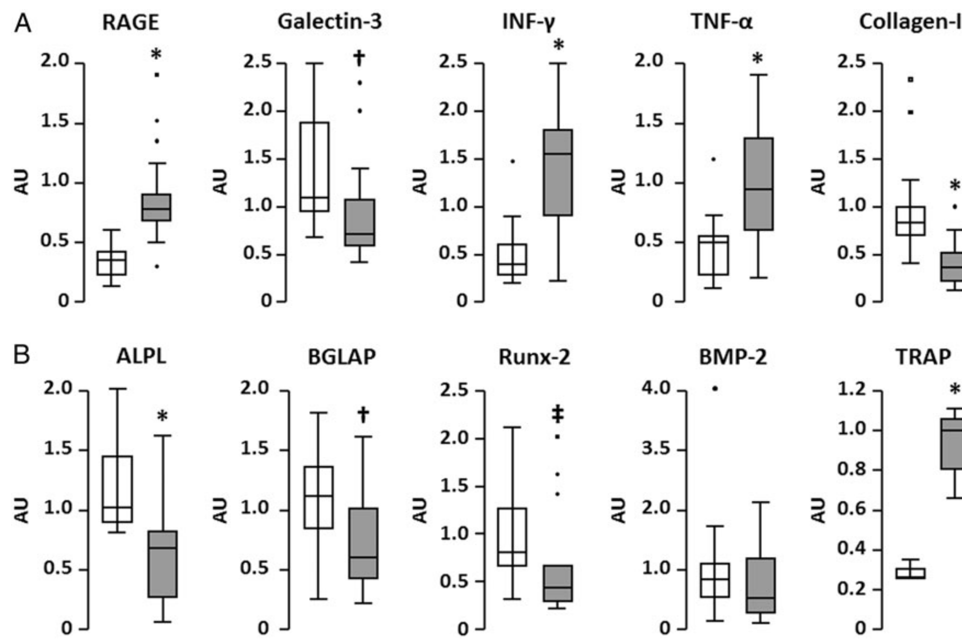

Figure 2 Gene expression levels of AGE receptors, inflammation and fibrosis markers (*A*) and osteogenic markers (*B*) in plaques with prevalent sheet-like/lamellated (macro, white bars) or spotty/granular (micro, grey bars) calcification [median (inter-quartile range); $n = 18$ each group]. $^{*}P < 0.001$, $^{\dagger}P < 0.01$ or $^{\ddagger}P < 0.05$ in macro vs. microcalcified plaques.

The above data also indicate that both receptors modulate VSMC osteogenic differentiation independent of ALEs/AGEs. However, since these by-products up-regulate both receptors and exert their injurious effects through RAGE,[11,22,28] we then evaluated the effect of CML on the process of osteogenic differentiation. Molecular analysis was limited to the first 2 weeks (Supplementary material online, *Figure S10*), since this is the longest incubation time of VSMCs with AGEs found in the literature[29] and also because at 3 weeks the mRNA expression data became irreproducible, possibly due to the cytotoxic effect of prolonged exposure to CML. Time course experiments in WT-VSMCs showed an early (1-week) pro-osteogenic effect of CML, which induced up-regulation of Runx-2, but not of ALPL, when compared with untreated VSMCs. Interestingly, also galectin-3 and RAGE expression was induced by CML treatment, whereas TRAP was not affected at this time. In contrast, at 2 weeks, while the stimulatory effect of CML on Runx-2 expression was maintained, ALPL was down-regulated, when compared with untreated WT-VSMCs. Galectin-3 expression showed a trend towards normalization, whereas RAGE and TRAP showed an opposite behaviour, being up-regulated by CML. In *Lgals3*$^{-/-}$-VSMCs, the initial pro-osteogenic effect of CML observed in WT cells was completely lost, since CML treatment did not modify Runx2 expression and down-regulated ALPL expression when compared with untreated cells, at variance with WT-VSMCs. At 2 weeks, CML treatment of *Lgals3*$^{-/-}$-VSMCs induced marked reduction of Runx2, whereas ALPL expression remained lower than in untreated cells. In contrast, RAGE and TRAP were up-regulated vs. untreated cells. CML treatment of siRAGE-*Lgals3*$^{-/-}$ monolayers produced intermediate effects between those observed in WT- and *Lgals3*$^{-/-}$-VSMCs (not shown). In response to CML, β-catenin translocation and Axin2 mRNA levels were reduced in WT cells, whereas nuclear β-catenin levels were unchanged and the expression of β-catenin target genes was further reduced in *Lgals3*$^{-/-}$-VSMCs (*Figure 5*). CML also caused an increase in apoptosis and a reduction in proliferation/viability in WT- and, to a higher extent, in *Lgals3*$^{-/-}$-VSMCs (Supplementary material online, *Figure S9*).

3.2.2 Mineralization pattern

Morphometric analysis of monolayers stained with Von Kossa (*Figure 6*) revealed an altered mineralization process in *Lgals3*$^{-/-}$-VSMCs, with a non-significant reduction of total calcified area and a more sparse distribution of calcification nodules, which were more numerous but of smaller size than in WT cells. These changes were only partly affected by RAGE silencing.

CML addition to WT-VSMCs induced a non-significant reduction of total calcified area, an increased number of calcifying nodules and a reduction of nodule size. Moreover, exposure to CML exacerbated the alteration of mineralization observed in untreated *Lgals3*$^{-/-}$-VSMCs, with further significant reduction of total calcified area and formation of significantly more numerous and smaller nodules. CML treatment also worsened mineralization of *Lgals3*$^{-/-}$-siRAGE cells.

Interestingly, despite the differences in total calcified area reflecting differences in calcification pattern, total calcium contents of monolayers, as measured colourimetrically, did not differ across the experimental conditions (not shown). This suggests that the galectin-3/RAGE dyad affects the arrangement rather than the extent of mineralization, depending on both the absolute and relative levels of these receptors.

4. Discussion

Carotid artery calcification is an unfavourable finding evidencing advanced atherosclerosis. However, previous reports have shown that extensive calcification could have a stabilizing influence on the plaque, entailing partial protection from thrombo-embolic events. In *ex vivo* studies, Hunt *et al.*[17] found that stroke and TIA occurred less

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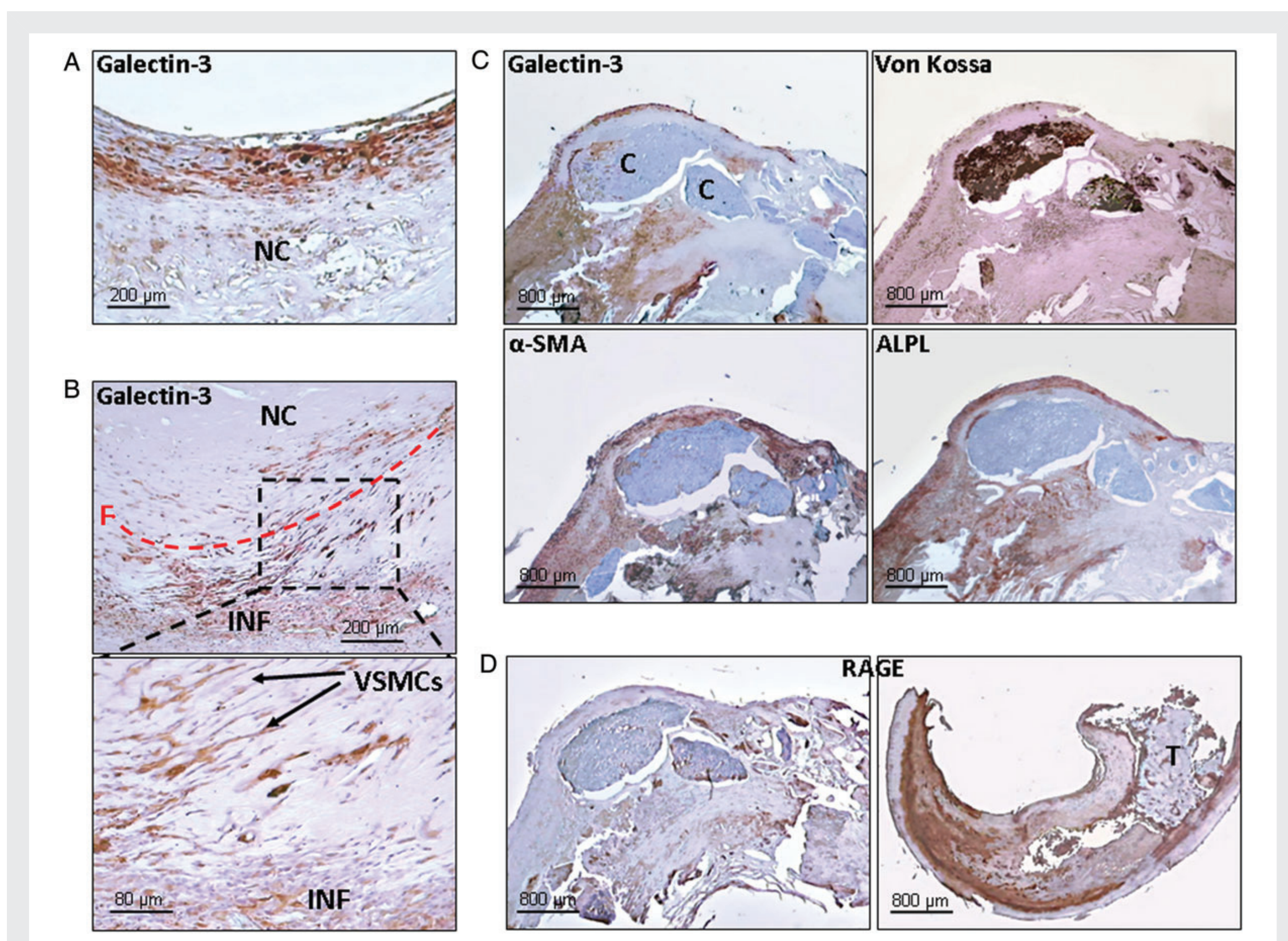

Figure 3 Immunohistochemistry for galectin-3, α-SMA, ALPL, and RAGE, and Von Kossa staining of representative plaques. Galectin-3 is expressed by lipid-laden cells (*A*, brown staining) and mononuclear cell infiltrates (INF) in the media and adventitia (*B*). In addition, also VSMCs stain for galectin-3, particularly in fibrous areas (*F*) surrounding the necrotic core (NC) (*B*). Moreover, intense galectin-3 positivity is observed near the large areas of sheet-like/lamellated calcification, with a staining pattern similar to that of α-SMA and ALPL (*C*). In contrast, RAGE positivity is negligible in macrocalcified plaques (*D*, left), whereas it is intense in microcalcified, inflamed and unstable plaques [*D*, right, same plaque as in (*A*) of *Figure 2*; T = thrombosis].

frequently in patients who had plaques with large sheet-like calcifications, and Shaalan *et al.*[30] showed that symptomatic plaques were less calcified and more inflamed than asymptomatic plaques. Moreover, an *in vivo* study using multidetector computed tomography showed that the extent of carotid plaque calcification was inversely associated with the occurrence of symptoms.[31]

In this study, we further support this concept by showing that sheet-like/lamellated (macro) and spotty/granular (micro) calcification are associated with different plaque phenotypes at both the molecular and morphological level. More importantly, we provide evidence that this association between type of calcification and plaque phenotype is related to the expression pattern of two AGE/ALE-binding proteins, galectin-3 and RAGE. In fact, galectin-3 was found to be expressed not only by inflammatory cells in unstable plaque regions, but also by VSMCs in areas of fibrosis and particularly of sheet-like/lamellated calcification indicating a more stable pattern. Nachtigal *et al.*[32] also reported that galectin-3 was expressed by VSMCs from CEA specimens, though it was mainly confined to foam cells. In addition, we showed that, in VSMCs adjacent to extensively and homogenously mineralized regions from fibro-calcific plaques, galectin-3 co-localized with the osteogenic marker ALPL. Conversely, expression of RAGE and its ligands CML and S100A12 prevailed in unstable, inflamed plaques with a prevalent spotty/granular calcification pattern. From an integrated viewpoint, the diverging relationships between galectin-3 and RAGE with sheet-like/lamellated and spotty/granular calcification, respectively, reflect the dual mode by which vascular calcification correlates with both inflammation and plaque stability. We also showed that these two receptors display diverging effects on VSMC osteogenic differentiation. In fact, this process was impaired in terms of molecular changes, induction of proliferation and pattern of mineralization in galectin-3-deficient VSMCs, in which RAGE was up-regulated. However, RAGE silencing restored only partly the osteogenic differentiation in these cells. In fact, galectin-3 was shown to exert a direct modulation of Wnt/β-catenin, a key event in osteogenic differentiation.[26] Moreover, these effects were independent of the AGE/ALE-binding function, though CML mimicked or aggravated the impact of galectin-3 ablation on VSMC differentiation through induction of RAGE expression and activation. Thus, accumulation of AGEs/ALEs

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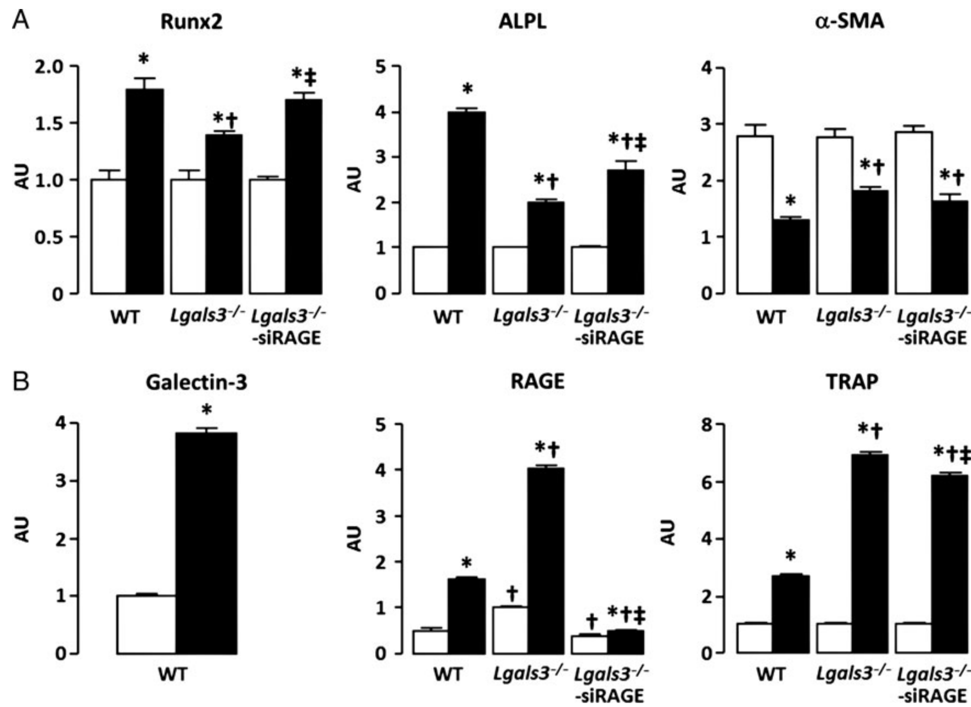

Figure 4 Gene expression levels of Runx2, ALPL, α-SMA, galectin-3, RAGE, and TRAP in VSMCs from WT and *Lgals3*$^{-/-}$ mice and in *Lgals3*$^{-/-}$ cells silenced for RAGE, grown in normal medium (white bars) or osteogenic medium (black bars) for 3 weeks ($n = 3$ in each of three independent experiments). *$P < 0.001$ vs. normal medium; †$P < 0.001$ vs. WT; ‡$P < 0.001$ vs. *Lgals3*$^{-/-}$.

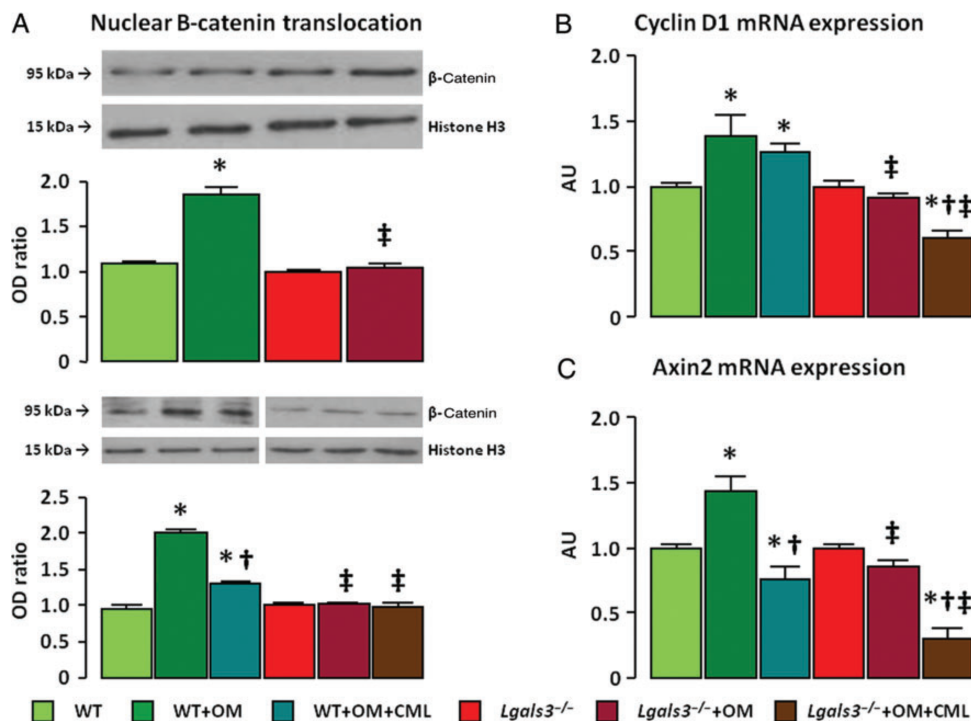

Figure 5 Nuclear β-catenin translocation at 24 h (*A*, top) and 1 weeks (*A*, bottom) and gene expression levels of cyclin D1 (*B*) and Axin2 (*C*) at 1 week in VSMCs from WT and *Lgals3*$^{-/-}$ mice grown in normal vs. osteogenic medium (OM) ± CML (100 μg/mL) ($n = 3$ in each of three independent experiments). *$P < 0.001$ vs. normal medium; †$P < 0.001$ vs. OM; ‡$P < 0.001$ vs. WT.

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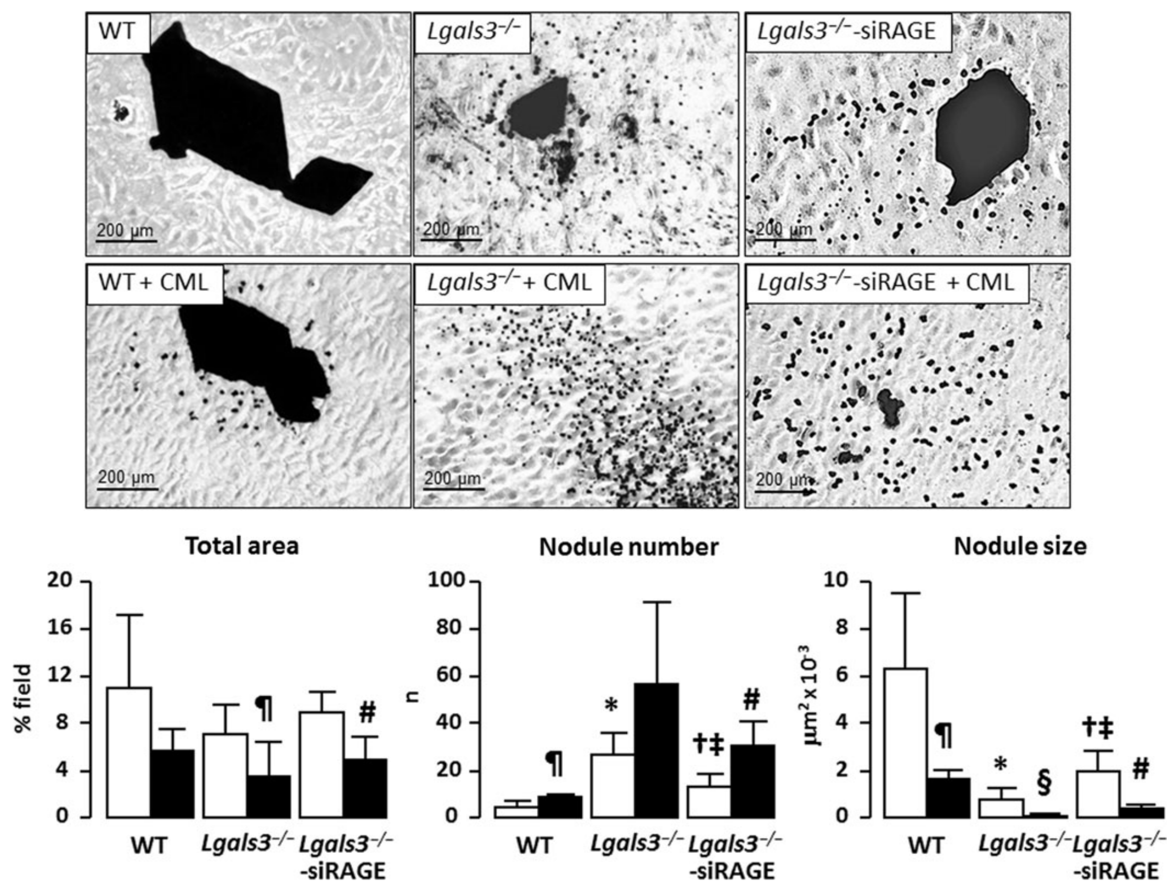

Figure 6 Von Kossa staining from representative monolayers and quantification of total calcified area, and calcified nodule number and size in VSMCs from WT and *Lgals3*$^{-/-}$ mice and in *Lgals3*$^{-/-}$ cells silenced for RAGE, grown in osteogenic medium, either untreated (white bars) or treated (black bars) with CML (100 μg/mL), for 3 weeks ($n = 3$ in each of three independent experiments). $^{*}P < 0.001$ or $^{\dagger}P < 0.01$ vs. WT cells; or $^{\ddagger}P < 0.01$ vs. *Lgals3*$^{-/-}$ cells; $^{\S}P < 0.001$, $^{\#}P < 0.01$ or $^{¶}P < 0.05$ vs. untreated cells.

and/or impaired galectin-3-dependent removal would favour dysregulation of vascular calcification mediated by the AGE-RAGE axis, leading to spotty/granular calcification and, hence, plaque instability.

These findings introduce a novel molecular mechanism underlying the biological activity of the galectin-3/RAGE dyad in atherogenesis, i.e. these two receptors modulate the pattern of plaque calcification through direct and diverging actions on vascular osteogenesis, in addition to regulating inflammation in opposite ways. In fact, galectin-3 and RAGE showed different effects on the induction of differentiation markers and organization of the mineralization process in VSMCs exposed to osteogenic medium. Galectin-3 is needed to acquire a complete osteoblast-like phenotype by VSMCs, consistent with the intranuclear localization of galectin-3 and its role in β-catenin activation at this level. This is in keeping with the findings that, in skeletal tissue, galectin-3 is a downstream target of Runx2, the key regulator of osteogenic differentiation,[14] and participate in the process of endochondral bone formation,[33] whereas it suppresses up-regulated osteoclastogenesis.[34] This is also consistent with the role of Wnt/β-catenin signalling in osteoblastogenesis[26] and the regulation of this pathway by galectin-3.[27] In contrast, RAGE appears to hamper VSMC osteoblatogenic differentiation and orderly calcium deposition. Altogether, our data point to the importance of galectin-3 and RAGE in determining plaque vulnerability and clinical outcomes by affecting both vascular osteogenesis and inflammation, which in turn triggers calcium deposition within the vessel wall. In plaques, RAGE could promote and perpetuate inflammation, thus favouring deposition of spotty/granular calcifications (microcalcifications) and hence plaque instability. Moreover, since RAGE was shown to be essential for osteoclast maturation and function in skeletal tissue both *in vivo* and *in vitro*[15] and osteoclasts were hypothesized to originate from monocytes–macrophages within the vessel wall,[35] it is still unclear whether RAGE affects vascular calcification also by inducing osteoclast differentiation and activation. Finally, immunohistochemistry failed to detect VSMC RAGE expression and its variation with plaque phenotype, due to the low sensitivity of the technique. However, the *in vitro* results in cells lacking galectin-3 or exposed to CML prompt the speculation that, though RAGE is expressed at low levels in VSMCs, up-regulation of this receptor might also counteract the osteoblastogenic effect of galectin-3, possibly via inhibition of β-catenin activation.[28] This view is in keeping with the observation that, in ApoE null mice, VSMC-targeted expression of the RAGE ligand S100A12 resulted in an increased number of calcific nodules and features of plaque instability.[13] Conversely, galectin-3, in addition to exerting an anti-inflammatory action, once the calcification process has started, would drive progression of calcium deposition within the vessel wall towards a pattern of sheet-like/lamellated calcification (macrocalcification).

Our data might also contribute to explain the effects of AGEs/ALEs on calcification. In fact, previous studies reported that VSMC osteogenic differentiation is stimulated[35,36] by modified lipoproteins and ALEs/AGEs,

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at variance with osteoblast growth and function, which are inhibited.[36,37] However, we found that CML, a major RAGE ligand, did favour the initial switch of VSMCs towards an osteogenic phenotype, consistent with previous reports,[35,36] but at a later stage it induced a dysregulated expression of osteogenic markers associated with disorganized mineralization. These findings suggest that the different effects of ALEs/AGEs on vascular and bone cells are only apparent, actually reflecting differences in cell commitment. Thus, in vessels, ALE/AGE accumulation would result in disorganized tissue mineralization favouring plaque vulnerability, consistent with the finding that oxidized lipoproteins destabilized calcific nodules formed by calcifying vascular cells implanted subcutaneously in ApoE-null mice.[38]

In conclusion, these results indicate that, in addition to their opposite effects on inflammation, galectin-3 and RAGE regulate, in diverging ways, vascular osteogenesis, by modulating Wnt/β-catenin signalling. They govern formation of sheet-like/lamellated and spotty/granular calcification, and the consequent plaque progression towards a stable or unstable phenotype, respectively. This represents a novel molecular mechanism underlying the activity of these ALE/AGE-receptors in the process of atherogenesis, even beyond their ALE/AGE-binding properties.

Acknowledgements

The Authors thank Cinzia Cataldo for technical assistance in histology processing and Valentina Della Chiara for patients' recruitment and clinical evaluation.

Conflict of interest: none declared.

Funding

This work was supported by grants from 'La Sapienza' University and the Diabetes, Endocrinology and Metabolism Foundation, Rome, Italy. Valentina Della Chiara is recipient of a fellowship from Laboratori Guidotti, Pisa, Italy.

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