Osteo/Chondrocytic Transcription Factors and Their Target Genes Exhibit Distinct Patterns of Expression in Human Arterial Calcification

Kerry L. Tyson, Joanne L. Reynolds, Rosamund McNair, Qiuping Zhang, Peter L. Weissberg, Catherine M. Shanahan

Objective—Mineralization-regulating proteins are found deposited at sites of vascular calcification. However, the relationship between the onset of calcification in vivo and the expression of genes encoding mineralization-regulating proteins is unknown. This study aimed to determine the temporal and spatial pattern of expression of key bone and cartilage proteins as atherosclerotic calcification progresses.

Methods and Results—Using reverse transcription-polymerase chain reaction on a panel of noncalcified and calcified human arterial samples, two classes of proteins could be identified: (1) Matrix Gla protein, osteonectin, osteoprotegerin, and aggrecan were constitutively expressed by vascular smooth muscle cells (VSMCs) in the normal vessel media but downregulated in calcified arteries whereas (2) alkaline phosphatase, bone sialoprotein, osteocalcin, and collagen II were expressed predominantly in the calcified vessel together with Cbfa1, Msx2, and Sox9, transcription factors that regulate expression of these genes. In the calcified plaque in situ hybridization identified subsets of VSMCs expressing osteoblast and chondrocyte-like gene expression profiles whereas osteoclast-like macrophages were present around sites of calcification.

Conclusions—These observations suggest a sequence of molecular events in vascular calcification beginning with the loss of expression by VSMCs, of constitutive inhibitory proteins, and ending with expression by VSMCs and macrophages of chondrocytic, osteoblastic, and osteoelastic-associated proteins that orchestrate the calcification process. (Arterioscler Thromb Vasc Biol. 2003;23:489-494.)

Key Words: calcification • atherosclerosis • osteoblast • cartilage • vascular smooth muscle cell • macrophage

Vascular calcification occurs as a complication of atherosclerosis and involves the nucleation of hydroxyapatite (HA) on membrane-bound vesicles and the local expression/deposition of bone-associated, mineralization-regulating proteins.1–3 Thus, it shares fundamental similarities with developmental osteogenesis and a feature of many end-stage calcified lesions is the presence of bone trabeculae and/or cartilage-like cells in the vessel wall.4,5 Until recently little was known of the function of bone-associated proteins in the vasculature, but gene knockout (KO) and in vitro studies have demonstrated that many of them regulate vascular smooth muscle cell (VSMC) phenotype and/or inhibit HA crystal growth.6–8 Moreover, the vascular phenotypes of the matrix Gla protein (MGP) and osteoprotegerin (OPG) KOs suggest that, in the normal vascular media, calcification is actively inhibited.6,9

Studies of human medial calcification (Monckeberg’s sclerosis) in diabetes and aging have suggested that the VSMCs that predominate in these lesions lose expression of calcification inhibitors, such as MGP, and begin to express “late” differentiation markers of both osteoblasts (bone sialoprotein; BSP) and osteocalcin (bone Gla protein; BGP) and chondrocytes (collagen II; COLII).4 This implies that vascular calcification may be caused by phenotypic modulation of resident vascular cells in a permissive matrix environment. However, few studies have explored the phenotype of VSMCs as calcification progresses in atherosclerotic lesions. Bone matrix proteins have been identified at sites of calcification, but it is unclear whether these were expressed locally by VSMCs or deposited from the circulation.2,3 Moreover, in contrast with Monckeberg’s sclerosis, macrophages associate with calcification in atherosclerotic plaques and express the mineralization inhibitor osteopontin, suggesting they may also play a role in regulating mineral deposition.10

In vitro studies using both bovine and human VSMCs have shown that VSMCs assume osteo/chondrocytic-like properties whereby they spontaneously, or in response to exogenous factors, co-express numerous osteoblast and chondrocyte markers, form nodules, and calcify over a defined time-course.4,11 The factors that regulate this phenotypic transition
are unknown; however, a number of potent transcription factors have been identified that regulate expression of many of the osteoblast and chondrocyte markers induced. These include Cbfal, the master regulator of bone differentiation and also essential for the induction of hypertrophic chondrocytes; Msx2, a homeobox-containing transcription factor crucial for cranial bone development; and Sox9, a master regulator of cartilage differentiation. The transcriptional targets of these factors include the bone proteins osteopontin (OP), BSP, BGP, and COLI for Cbfal; BGP for Msx2; and the cartilage proteins aggrekan (AGG) and COLII for Sox9. The observation that Cbfal is expressed in VSMCs in vitro suggests that it may be involved in regulating the osteo/chondrocytic transition of VSMCs in vivo. Cbfal has also been identified in the calcified aorta of MGP KO mice. However, cartilaginous metaplasia of the aorta was the most prevalent feature in these mice. To date, the role of cartilage-specific transcription factors, such as Sox9, in calcification and the osteo/chondrocytic conversion of VSMCs has not been explored.

In this study we hypothesized that progressive changes in expression of genes encoding bone- and cartilage-associated proteins may be involved in the regulation of vascular mineralization and/or VSMC phenotypic change. We examined expression of a group of bone and cartilage markers, including late differentiation markers and their corresponding regulatory transcription factors, in normal arteries or arteries containing only fatty streaks (both noncalcified), and in arteries with early and advanced atherosclerotic lesions (all calcified). We demonstrate that before calcification, the expression of inhibitors of mineralization was downregulated whereas in the calcified plaque, both VSMCs and macrophages exhibited phenotypic characteristics indicative of cells capable of actively regulating mineral deposition and re-absorption.

**Materials and Methods**

**Collection of Material**

Human aortic (n=30) and carotid endarterectomy specimens (n=20) were obtained at surgery with appropriate ethical approval. The degree of atherosclerosis was assessed by viewing en face, and a representative piece of each vessel was excised and snap frozen for histological analyses to accurately stage lesions and for use in in situ hybridization (see online Table I, which can be accessed at http://atvb.ahajournals.org).

**Immunohistochemistry**

Immunohistochemistry was performed on frozen sections using an avidin–biotin reaction kit (Dako) according to the manufacturer’s instructions. Sections adjacent to those used for in situ hybridization were stained for α-SM actin (smooth muscle), CD68 (macrophages), CD3 (T-cells), and CD31 (endothelial cells). Lipid and calcium were viewed using Oil Red O and von Kossa, respectively.

**RNA Preparation**

The adventitial and endothelial cell layers were removed from each vessel and the remainder was enzyme dispersed in 3 mg/mL collagenase/1 mg/mL elastase (Sigma) and washed twice in ice-cold PBS. Explant cultures were established as previously described and cells were harvested before passing. RNA was prepared from tissue or cell pellets and was DNase digested at 37°C for 1 hour to remove contaminating DNA before preparation of cDNA.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA (5 μg) was reverse-transcribed for 1 hour at 42°C using AMV reverse transcriptase and oligo-dT primer. To ensure that amplification was within the linear range, test reactions at 20, 22, 25, 30, and 35 cycles were performed and products were Southern blotted, hybridized, and quantitated. For all subsequent reactions, 2.5 μL of cDNA was used in 20-μL reactions using the primers and conditions described (see online Table II). Primer pairs covered an intron so that the size of the PCR amplicon showed that only cDNA was amplified whereas the identity of each PCR product was confirmed by sequencing. Reactions were performed twice and a negative (no RT) and positive control (SAOS2 cDNA) included with each reaction set. PCR products were electrophoresed on a 1% agarose gel and Southern blotted before hybridization with the appropriate 32P-labeled probe. Quantitation was performed by counting in real time on an InstantImager (Packard) and standardized to a β-microglobulin control. Statistical analysis was conducted using a Student’s t test and ANOVA.

**In Situ Hybridization**

Sections were cut (6 μm) from carotid endarterectomy specimens and processed for in situ hybridization as previously described. Briefly, sections were hybridized overnight at 50°C using 2×106 cpm/slide of 32P-labeled sense or antisense riboprobes in 200 μL of hybridization solution. Probes were of similar size and included cloned PCR fragments (see Table II) and cDNAs. Slides were sequentially washed and then left to expose to Ilford K5 emulsion for 3 weeks in the dark at 4°C before being developed, counterstained with hematoxylin-eosin, and mounted.

**Results**

**Transcription Factor Expression in Osteo/Chondrocytic VSMC In Vitro**

Human VSMCs in vitro undergo a spontaneous osteo/chondrocytic conversion and begin to co-express markers of both osteoblastic (ALK, BSP, and BGP) and chondrocytic (COLII) lineages. To determine the transcriptional pathways that are activated concomitantly with this in vitro conversion, we used RT-PCR to examine expression of master regulators of bone (Cbfal, Msx2) and cartilage (Sox9) differentiation before and after culture. Freshly dispersed normal medial VSMCs expressed undetectable levels of Cbfal, Sox9, and Msx2; however, in vitro VSMCs clearly upregulated expression of Cbfal and Sox9 (Figure 1). Msx2 expression was also detectable in vitro but only after hybridization of the PCR product, suggesting it was expressed at low levels (not shown). Thus, the osteo/chondrocytic conversion of VSMCs in vitro is associated with activation of multiple transcriptional regulatory pathways.
RT-PCR Analysis of Gene Expression in Noncalcified and Calcified Arteries

To determine whether similar changes took place in vivo, we undertook RT-PCR analysis of gene expression in noncalcified and calcified arteries. RT-PCR analysis of gene expression in normal, noncalcified arteries revealed that OPG, MGP, secreted protein, acidic, cystein-rich/osteonectin (SPARC), OP, and AGG were constitutively expressed. Expression of ALK, BSP, BGP, COLII, and the transcription factors Msx2 and Cbfa1 was low to undetectable, whereas Sox9 and COLI, were variably expressed (Figures 2 and 3). In noncalcified arteries with fatty streak lesions expression of MGP and SPARC expression was significantly reduced, whereas the expression patterns of the other genes were unchanged. Expression of SM22α, a smooth muscle (SM)-specific differentiation marker, was not significantly reduced in fatty streak lesions, suggesting that reduced expression of MGP and SPARC was not caused by changes in SM abundance (not shown).

In marked contrast, expression of ALK, BSP, BGP, Cbfa1, and Msx2, all markers of osteogenic differentiation, was significantly induced in calcified vessels. COLII, a marker of chondrocytic differentiation, was also highly expressed in some calcified lesions. COLI and Sox9 were highly variable in their expression, as seen in the noncalcified vessels; however, the highest Sox9 expression was observed in calcified carotid lesions (Figures 2 and 3). Vessels in each sample group were from males and females across a wide range of ages but there was no evidence for changes in expression of bone-associated proteins with age, only with disease and calcification.

In Situ Hybridization

To obtain a detailed profile of gene expression within the calcified plaque, nine carotid lesions were used for in situ hybridization and immunohistological analysis. In these lesions calcification invariably colocalized with lipid and although intimal calcification was most prevalent, in some lesions, the media beneath large atherosclerotic plaques was also calcified (see online Figure I). Medial calcification was exclusively associated with VSMCs, whereas in intimal areas both VSMCs and/or macrophages were present. Macrophages were most abundant in association with calcification of large lipid pools whereas intimal VSMCs were associated with isolated areas of fine granular calcification in matrix-rich areas (Figure 4 and online Figure II). In none of the lesions was there morphological evidence of bone or cartilage formation.

In situ hybridization demonstrated that within the plaque, in association with both medial and intimal calcification, subsets of α-SM actin–positive VSMCs expressed ALK, BSP, and BGP, which is indicative of osteogenic conversion. In the same areas, expression of MGP, OP, and SPARC was low and/or patchy although expression of SM22α was detectable (Figure I). Cells expressing ALK, BSP, and BGP generally did not express COLII, a marker of chondrocytic conversion. COLII was expressed patchily in intimal and medial VSMCs not always in association with calcification (Figure 4). Expression of the transcription factors Cbfa1 and Sox9 was undetectable using in situ hybridization. Msx2 expression was detected in isolated calcified intimal VSMCs and in microvessels in the lesion intima and adventitia, areas that were generally not calcified (Figure 4).

In association with lipid pools and granular calcification, subsets of CD68-positive macrophages co-expressed high levels of ALK, BSP, BGP, and OP. Some macrophages associated with calcification also expressed lower levels of MGP, SPARC, and COLI (Figure II).

Discussion

In this study we showed that the osteo/chondrocytic conversion of VSMCs in vitro correlated with expression of Cbfa1, Sox9, and Msx2, master regulators of bone and cartilage differentiation. Thus, in vitro, phenotypically modified calcifying VSMCs activate a number of mesenchymal transcriptional pathways that are suppressed in fully contractile, differentiated cells. Msx2 can modify the activity of both Cbfa1 and Sox9, and these transcription factors have a number of target genes in common.20,21 Therefore, it is likely that these factors act in concert to regulate VSMC phenotype at least in vitro.

Loss of MGP Expression May Initiate VSMC Phenotypic Modulation

Next, we looked in vivo to determine whether there was evidence for similar changes in gene expression during the progression of atherosclerotic calcification assuming a temporal progression from normal vessel to fatty streak to calcified atherosclerotic lesion. We found that VSMCs in the normal vessel wall expressed a number of inhibitors of mineralization, including MGP, that were downregulated in uncalcified fatty streaks and/or calcified lesions. MGP was

![Figure 2. Representative RT-PCR showing expression of bone-associated proteins in 5 of 20 noncalcified (samples 1 to 20) and 5 of 20 calcified vessels (samples 21 to 40). Selected lesions demonstrate the range of expression levels within both sample groups. β-microglobulin is representative of equivalent loading of samples. See Table I for definition of lesion numbers.](http://atvb.ahajournals.org/content/491/5/491/F2.large.jpg)
initially thought to inhibit calcification purely through binding to HA. However, a number of observations suggest that MGP may also regulate VSMC differentiation. For example, MGP KO mice develop cartilaginous metaplasia, and overexpression of MGP can inhibit osteochondrocytic conversion of mesenchymal stem cell lines.\(^8,22\) Furthermore, MGP can bind BMP2, which is a modulator of VSMC phenotype.\(^23\) Thus, loss of MGP may “prime” VSMCs for phenotypic change by allowing normally inactivated morphogens to act on the vessel wall. In support of this, BMPs have been shown to be deposited in the vessel wall in association with calcification. Moreover, BMPs can potently induce expression of Cbfal, Sox9, and Msx2, transcription factors that are upregulated in the calcified vasculature.\(^3,11,24,25\) Thus, MGP may play a greater role in dictating VSMC phenotypic expression that has been previously recognized.

**VSMCs Exhibit Osteogenic and Chondrocytic Phenotypes in Calcified Arteries**

In calcified atherosclerotic plaques, a dramatic shift in gene expression occurred. Expression of ALK, BSP, BGP, and COLII
was increased together with Cbfa1, Sox9, and Msx2, the transcription factors that regulate their expression. This strongly suggests that in calcified atherosclerotic plaques VSMCs reactivation the same mesenchymal transcriptional program we observed in vitro. Also, in situ hybridization confirmed that some α-SM actin-positive intimal VSMCs in a matrix-rich region of the plaque expressing Msx2 (arrows in left panels) and α-SM actin-positive VSMCs in the media/intima expressing COLII (arrows in right panels). AS (antisense probe) and S (sense control). M, media delineated by arrows; In, intima; Lu, lumen. The IEL is shown by arrowheads in lesion and arrows in right panels.

Our study is the first to report expression of Sox9 in the vasculature. Sox9 is a master regulator of cartilage differentiation that functions upstream of Cbfa1 in chondrocyte differentiation and bone formation. It was expressed in noncalcified and calcified arteries and potentially regulates expression of a number of matrix components common to both the vasculature and cartilage, such as AGG. In a number of carotid lesions both COLII and Sox9 were highly expressed. Although we could not directly demonstrate Sox9 and COLII expression in the same cells, VSMCs expressing COLII were observed in calcified lesions, suggesting a chondrocytic conversion. Interestingly, these cells generally did not colocalize with those expressing osteoblastic markers, suggesting that locally derived morphogens may dictate which particular transcriptional pathway will be dominant in VSMCs. For example, there is evidence to suggest that transforming growth factor-β1 can mediate chondrocytic conversion of VSMCs in vivo and this morphogen has been shown to localize to specific regions of the plaque during atherosclerotic progression.

Msx2, a transcription factor crucial for cranial bone development, was also expressed by isolated intimal VSMCs and microvessels. Previously, in a model of diet-induced diabetes, in LDL receptor-deficient mice, Msx2 was shown to be upregulated in adventitial microvessels. It was suggested that pluripotent cells, such as pericytes of the adventitia, undergo Msx2-regulated osteogenic conversion and these cells migrate into the vessel media and contribute to calcification. Our study would support the notion that within human atherosclerotic plaques, microvessels serve as conduits for the invasion of “mesenchymal stem cells,” which may form bone, generally a late event in calcification. Indeed, in cardiac valve calcification, neangiogenesis is a prerequisite for lamellar bone formation.

**Macrophages Exhibit Osteoclastic Features in Calcified Arteries**

We identified subsets of macrophages, only in calcified arteries, that expressed high levels of OP, BSP, ALK, and BGP. This expression profile is typical of macrophages that can be isolated from the circulation, tissues, and breast tumors and that have the capacity to differentiate into osteoclast-like cells in vitro and in animal models. This osteoclastic expression profile was not found in fatty streaks that contain abundant macrophages, suggesting that osteoclast induction is dependent on a calcified environment. Moreover, OPG a member of the tumor necrosis factor-α receptor family with a well-defined role as an inhibitor of osteoclast differentiation, was downregulated in the calcified vessel wall, and OPG protein has been shown previously to be absent from sites of calcification. Thus, loss of OPG may be permissive for osteoclastic differentiation of macrophages. Descriptions of remodeled bone in the vessel wall suggest that osteoclastic activity must be present, at least at late stages of calcification/ossification, and our study adds further to the extensive evidence supporting the presence of osteoclastic macrophages in atherosclerotic plaques. Functional studies are now required to examine the HA-reabsorbing properties of plaque macrophages.

In summary, this study has identified a number of bone-associated proteins that change expression as vascular calcification progresses and that, by inference from their roles in endochondral ossification, are key regulators of vascular calci-
stitution. Our data suggest that VSMCs normally express proteins that inhibit calcification but that in early atherosclerosis and before calcification, expression of some of these inhibitors is downregulated, contributing to a loss of homeostatic inhibition of calcification and, potentially, changes in VSMC phenotype. In established calcification, there is evidence for activation of both chondrocytic and osteogenic transcriptional pathways in VSMCs, coupled with activation of an osteoclastic pathway in macrophages. The local environmental factors responsible for these events remain to be identified.

Acknowledgments
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References
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**Table I**: Aortic and carotid samples examined.

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</table>

Stary classifications are shown. Calcification was assessed by von Kossa staining. nd, none detectable; s, macrophages; I/M, intima/media ratio.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5'-3')</th>
<th>T (°C)$^5$ (Cycle no.)</th>
<th>Size*$^*$</th>
</tr>
</thead>
</table>
| β-micro | F: GATTCAGGTTTACTCACG  
R: CCATGATGCTGCTTACATG | 55 (22) | 294bp |
| SM22α | F: ATGGGCAAACAAGGCTCCATC  
R: ACTGATGATCTGCGGCGGT | 55 (25) | 601bp |
| OP | F: ACTGATTTTCCCCAGGAC  
R: ATGGCTGTGGAATTACG | 58 (30) | 428bp |
| MGP | F: ATGAAGAGCCTGATCCTTCTT  
R: TCATTTCCTCCCTCGGCTT | 55 (25) | 312bp |
| ALK | F: ACTGTTGCTAAAGATGTCATC  
R: CTGGTAGCCGATGTCCCTA | 58 (35) | 475bp |
| BSP | F: TGCTCAAGCTTTTTGGAAT  
R: TGCATTGCTCCAGTGACACT | 58 (35) | 627bp |
| BGP | F: GAGTCTCTGAGCAGCAGCCCAGC  
R: GGTGCTGGAGGAGGAGCAGA | 68 (35) | 408bp |
| SPARC | F: GACAATGACAAGTGACATGCCTCGCCTG  
R: CTCAGTTACAGCCTCAAGGCAAC | 55 (25) | 648bp |
| COL I(α1) | F: TGACGAGACAAGAAGACTGCC  
R: CCATCAGACAACCACTGGAACC | 58 (30) | 599bp |
| COL II(α1) | F: AACGTTCCCAGAGAAGAAGCTGG  
R: GTCCACACCCAGTCCCTGCT | 58 (35) | 454bp |
| AGG | F: ATGCCAAGACTCACCAGTGG  
R: GTCTGGAGAGCTCTTCCTTGCT | 59 (25) | 318bp |
| Cbfa1 | F: CCCACGACAAACCGCACCAT  
R: CACTCCGCGCCACAAATTC | 60 (30) | 297bp |
| Msx2 | F: GCAAGACATACGGACCTACCCAGCTCTGCT  
R: GGACAGGTGGTACATGTCCATATCC | 65 (35) | 400bp |
| Sox9 | F: AGAGCGGGAGGACAAGTTC  
R: CGTTCTTCACCAGCTTCTC | 62 (30) | 330bp |

Forward (F) and reverse (R) primers for PCR reactions. $^5$ shows annealing temperature used for each primer pair and * indicates the expected PCR product size if cDNA is amplified.
**Figure I:** *In situ* hybridization showing expression of bone-associated proteins in the calcified media beneath an atherosclerotic plaque from a 70 year old female. The boxed region is shown at higher power. This area was calcified (brown stain shows von Kossa (VK)) and contained α-SM actin positive VSMCs but no macrophages (CD68). *In situ* hybridization showed that ALK, BSP and BGP were co-expressed by VSMCs in the calcified media while levels of OP, MGP and SPARC were barely detectable in the same region. M, media delineated by arrow, arrowheads show internal elastic lamina (IEL); In, intima; FC, fibrous cap.
Figure II: *In situ* hybridization demonstrating expression of bone-associated proteins by macrophages. Boxed region in shoulder region of lesion (from a 78 year old male) is enlarged to demonstrate lipid filled (ORO stain), CD68 positive macrophages associated with a calcified (brown in VK) area, devoid of VSMCs. *In situ* hybridization demonstrates that ALK, BSP, BGP and OP are co-expressed by the macrophages in this region. Low expression of MGP and SPARC was found in other subsets of macrophages associated with calcification (in these panels arrows indicate autofluorescence of calcification, fine purple grains indicate positive hybridization). M, media delineated by arrows; In, intima; Lu, lumen.