Medial Localization of Mineralization-Regulating Proteins in Association With Mönckeberg’s Sclerosis
Evidence for Smooth Muscle Cell–Mediated Vascular Calcification

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Background—Calcification of the media of peripheral arteries is referred to as Mönckeberg’s sclerosis (MS) and occurs commonly in aged and diabetic individuals. Its pathogenesis is unknown, but its presence predicts risk of cardiovascular events and leg amputation in diabetic patients. Several studies have documented expression of bone-associated genes in association with intimal atherosclerotic calcification, leading to the suggestion that vascular calcification may be a regulated process with similarities to developmental osteogenesis. Therefore, we examined gene expression in vessels with MS to determine whether there was evidence for a regulated calcification process in the vessel media.

Methods and Results—In situ hybridization, immunohistochemistry, and semiquantitative reverse-transcription polymerase chain reaction were used to examine the expression of mineralization-regulating proteins in human peripheral arteries with and without MS. MS occurred in direct apposition to medial vascular smooth muscle cells (VSMCs) in the absence of macrophages or lipid. These VSMCs expressed the smooth muscle–specific gene SM22α and high levels of matrix Gla protein but little osteopontin mRNA. Compared with normal vessels, vessels with MS globally expressed lower levels of matrix Gla protein and osteonectin, whereas alkaline phosphatase, bone sialoprotein, bone Gla protein, and collagen II, all indicators of osteogenesis/chondrogenesis, were upregulated. Furthermore, VSMCs derived from MS lesions exhibited osteoblastic properties and mineralized in vitro.

Conclusions—These data indicate that medial calcification in MS lesions is an active process potentially orchestrated by phenotypically modified VSMCs.

Key Words: osteopontin ■ proteins ■ cartilage ■ muscle, smooth ■ diabetes mellitus

Vascular calcification is associated with increased risk of myocardial infarction, limb amputation, and morbidity after vascular surgery.1–5 It occurs at 2 anatomic sites: in the intima, where it is invariably associated with atherosclerosis, and in the tunica media.6 In atherosclerosis, intimal macrophages and vascular smooth muscle cells (VSMCs) express collagenous (collagen types I and IV) and noncollagenous bone matrix proteins (osteopontin [OP], osteonectin [SPARC]; secreted protein, acidic, rich in cysteine], matrix Gla protein [MGP], osteoglycin, and bone morphogenetic protein [BMP 2]), often in association with calcification.7–11 The association of intimal calcification with lipid, apoptotic cells, and matrix vesicles, important elements in bone development, suggests that intimal calcification is an active rather than a degenerative process, although the precise mechanism by which calcification is induced is poorly understood.4,12–17

Human medial calcification, Mönckeberg’s sclerosis (MS), is common and occurs independently of atherosclerosis, implying different etiological mechanisms.6,18 MS was originally described in aged males (>50 years); however, it is most commonly seen in diabetic individuals.19,20 The clinical significance of MS remains controversial; however, it is as an independent predictor of cardiovascular events in diabetic patients and is associated with trophic foot ulceration and peripheral artery occlusive disease.5,21–23

MGP is a 10-kDa circulating protein that contains 5 γ-carboxyglutamic acid residues that bind calcium in soft tissues.16,24 Mice lacking MGP develop extensive medial calcification and cartilaginous metaplasia, resulting in neonatal death by aortic rupture. We have previously shown that MGP is expressed by normal human medial VSMCs and is upregulated in the calcified atherosclerotic intima. However, its role in medial calcification in humans has not been elucidated.9 We hypothesized that medial calcification is a regulated process, and we determined the cellular pattern of expression of mineralization-regulating proteins in human...
MS lesions. Our findings suggest that in association with MS, medial VSMCs become modified and exhibit osteocytic/chondrocytic changes in gene expression, potentially conferring mineralization-regulating properties on them.

### Methods

#### In Situ Hybridization

Peripheral arteries from patients undergoing leg amputation (see Table) were snap-frozen and mounted in Tissue-Tek OCT embedding compound (Miles Ames Division, Inc). Sections (8 to 10 μm) were mounted onto gelatinized slides and processed for in situ hybridization as described previously. Human OP and MGP probes were transcribed from full-length cDNA clones of 1.4 and 0.7 kb, respectively (ATCC/NIH repository, Rockville, Md), whereas rat SM22α (3RF10) was from a 1.0-kb cDNA clone. Slides were exposed for 3 to 6 weeks, developed, stained with hematoxylin and eosin, and mounted.

#### Immunohistochemistry

Immunohistochemistry was performed with a Dako avidin-biotin immunoperoxidase kit according to the manufacturer’s instructions. VSMCs were identified with a mouse monoclonal antibody (Dako, M815, dilution 1:25) to human α-smooth muscle (α-SM) actin, and macrophages were identified with a mouse monoclonal antibody to CD68 (Dako, macrophage EMB11, dilution 1:20). A mouse monoclonal OP antibody (MPIIIB101) was obtained from the National Institute of Child Development Hybridoma Bank (Iowa City, IA) and diluted 1:200. The MGP polyclonal antibody (a gift from Dr R.F. Loeser, The Bowman Gray School of Medicine, Wake Forest University, Raleigh, NC) was raised in rabbits immunized with a 19-amino-acid synthetic peptide to a bovine MGP sequence as described and diluted 1:100. Controls were performed with the primary antibody substituted with either PBS or an irrelevant antibody. Calcium was demonstrated with von Kossa stain and lipid by oil red O staining.

#### Culture of Human VSMCs

Explants were cultured from peripheral arteries of 5 diabetic patients with MS and 5 control subjects (men and women aged 48 to 93 years). A piece of each vessel was retained for subsequent pathological examination. The endothelial and adventitial layers were removed, and medial smooth muscle cells directly in contact with...
calcified regions or calcium deposits alone were placed in 6-well plates with M199 media containing 20% fetal calf serum and antibiotic supplements. Six to 12 isolates were established from each vessel. Cell growth began after ~2 weeks, and at confluence, RNA was harvested from each well and cDNA prepared from 5 µg of total RNA. Wells left to become postconfluent were stained with von Kossa stain after ~30 days.26

Reverse-Transcription Polymerase Chain Reaction Analysis of Gene Expression in Human Arteries and Cultured Cells

mRNA was extracted from cultured cells or from 20 frozen sections cut from each vessel by lysis in buffer containing NP40, as previously described.11 A section was cut before and after the 20 sections for reverse-transcription polymerase chain reaction (RT-PCR) for pathological analysis. The RNA pellet was suspended in water and treated with DNase (1 hour at 37°C with 10 U of RNase-free DNase). Total RNA was reverse-transcribed for 1 hour at 42°C with avian myeloblastosis virus (AMV) reverse transcriptase and oligo-dT primer. To ensure that each PCR reaction was performed within the linear range of amplification, test reactions were performed for each primer pair at 20, 25, 30, 35, and 40 cycles. Southern blots were done on these reaction products, and they were hybridized and counted; plots of these results were used to establish linearity. Subsequently, 2.5 µL of cDNA was used in each 20-µL PCR reaction cycled for 30 to 35 cycles at 94°C for 2 minutes, annealing temperature for 1.5 minutes, and extension for 2 minutes at 72°C. Primers were designed from published human sequences in the Genbank/European Molecular Biology Laboratory databases, with the size of the PCR amplification product verifying that only cDNA was amplified. PCR products were sequenced or hybridized to a known cDNA. Reactions were performed twice, and positive (cDNA from human bone sections or SaOS cells) and negative (no cDNA) controls were included. PCR products were run on a 1% agarose gel, subjected to Southern blotting, and hybridized with appropriate 32P-labeled probes, then washed at high stringency. Quantification was performed by real-time counting on an Instant-Imager (Packard), and results were standardized to a β-microglobulin control.

Results

Cellular Composition and Calcium and Lipid Accumulation in MS Lesions

Peripheral arteries from consecutive amputations were taken from patients undergoing surgery because of gangrene in the lower limb (diabetic patients) or for ischemia related to atherosclerosis or trauma (nondiabetic patients) (Table). All diabetic patients exhibited extensive medial calcification, whereas only 1 older nondiabetic patient exhibited such calcification. In mildly affected arteries, medial calcification, associated with elastic fibers, was present throughout most of

Figure 1. A, Artery with mild MS (patient 17). Brown von Kossa staining localizes to elastic fibers and internal elastic lamina (arrowheads). B, Artery with advanced MS (patient 7) (arrowhead) and intimal calcification (arrow). C, Artery exhibiting amorphous medial calcification (arrow) and a region of bone (large arrowhead) (patient 6). Note osteocytes (small nonstaining holes) within bone and intimal calcification (small arrowheads). D, Oil red O staining of artery in B showing lipid accumulation exclusively in intima. Arrows indicate media (M). In indicates intima.
the medial width. In advanced lesions, the media was filled with circumferential rings of calcium, and at later stages, osteocytes were seen within bone trabeculae with apparent bone marrow formation (Figure 1). Calcification in the media occurred in the absence of macrophages and lipid. This contrasted with the scattered, globular calcification of the intima, which was invariably associated with lipid and macrophages (Figures 1 and 2). Medial calcification was found in association with α-SM actin–positive VSMCs, and significantly, calcium deposits were also present within apparently normal areas of VSMCs deep in the media (Figure 2). Moreover, these VSMCs expressed high levels of SM22α (Figure 3).

**Gene Expression and Protein Distribution of MGP and OP in Arteries**

MGP mRNA was detected throughout the media of normal arteries, but expression was low in the media of arteries with MS. However, in a small subset of VSMCs adjacent to medial calcification, MGP mRNA expression was highly elevated (Figure 3). MGP protein was present in the media of most arteries in a striated pattern, which suggests it was associated with the elastic lamina. However, in arteries with severe MS, this pattern was lost, and deposition was more diffuse and patchy (Figure 4).

High expression of OP mRNA was only detected in a subset of macrophages in atherosclerotic lesions (Figure 3). In normal vessels, OP protein was not detectable in the media, but it accumulated in MS lesions at the smooth muscle cell–calcium interface (Figure 2). OP protein surrounded all areas of calcification, including ossified regions of the media (not shown).

**RT-PCR Analysis of Bone-Associated Gene Expression in Arteries With and Without MS**

The above analyses indicated that medial calcification and ossification were closely associated with VSMCs. To evalu-

![Image](https://example.com/image.jpg)
ate the phenotype of VSMCs within MS lesions, semiquan-
titative RT-PCR analysis of cDNA derived from tissue
sections was performed with sections of arteries devoid of
atherosclerosis. Expression of SM22α, OP, and BMP2 was detectable in
both MS and normal vessels. However, despite the high
levels of MGP mRNA expressed by a subset of VSMCs,
overall, MGP mRNA expression was lower in vessels with
MS than in normal vessels (Figure 5). Normal vessels
expressed undetectable to low levels of alkaline phosphatase
(ALK), bone sialoprotein (BSP), and bone Gla protein (BGP).
In contrast, arteries with MS expressed significantly higher
levels of mRNA for all 3 proteins (Figure 5). The level of
expression of these genes did not correlate with the degree of
calcification. In situ hybridization confirmed that expression
of mRNA for these 3 proteins in MS lesions was at a low
level throughout the vessel media (not shown). VSMCs in
normal arteries express SPARC at high levels and collagen
(COL) II, a differentiation marker for chondrocytes, at low
levels. However, in MS arteries, there was a significant
reduction in the level of SPARC expression and a significant
increase in COL II expression. In contrast, there were no
differences in expression of COL I, which was variably
expressed (Figure 5).

Primary Culture of MS-Derived VSMCs
Primary explant cultures of VSMCs were established to
examine whether VSMCs derived from MS vessels could
mineralize in vitro. RT-PCR was used to determine the extent
of bone-associated gene expression in confluent, primary cell
cultures from isolates of VSMCs derived from both normal
and MS vessels. Gene expression in these VSMCs was
compared with that of SaOS-2 cells, an osteoblast-like cell
line.

At confluence, all primary cell explant cultures showed a
“hills-and-valleys” morphology, were α-SM actin positive,
and expressed SM22α (Figure 6A). In monolayer culture,
these cell isolates expressed MGP, COL I, COL II, and
SPARC at generally much higher levels than in SaOS-2 cells.
They also expressed ALK, BGP, OP, and BMP2, some at
higher levels than in SaOS-2 cells. Only BSP was expressed
at lower levels by VSMCs derived from MS vessels com-
pared with both VSMCs derived from normal vessels and
SaOS-2 cells (Figure 6C). After confluence, all VSMC
isolates formed multicellular, nodular condensations that
mineralized after 30 days (Figure 6B). Moreover, isolates of
each culture maintained these properties after multiple pas-
sages (not shown).

Discussion
Medial calcification is almost exclusively associated with
VSMCs in contrast to intimal calcification, which occurs in
macrophage- and lipid-rich atherosclerotic lesions. We have
found that in MS, VSMC expression of MGP and SPARC is
diminished, whereas expression of ALK, BSP, BGP, and
COL II is increased. This osteocytic/chondrocytic transfor-
mation of VSMCs suggests that medial calcification is a
regulated process that reflects either an adaptive response to
limit mineralization or an osteogenic response to facilitate it.

What Is the Role of Mineralization-Regulating
Proteins in the Vasculature?
Although evidence from the MGP knockout mouse suggests
that medial calcification in the developing artery is actively
inhibited by MGP, its role in adult human vessels is
unknown. Total expression of MGP was lower in vessels with
MS, which suggests that reduced MGP may predispose to
calcification. Its localized upregulation by VSMCs in MS
lesions may reflect an attempt to enhance calcium clearance.
In support of this concept, we have shown that when human
VSMCs calcify in vitro, MGP mRNA expression is upregu-

Figure 4. MGP protein accumulation in an early MS lesion
(patient 19). A, von Kossa stain shows low levels of calcification
in vessel media (brown). MGP protein (B) is deposited in media
but is absent from intima except in association with endothelial
cells. Arrowheads point to internal elastic lamina. M indicates
media; and In, intima.
lated at precisely the time hydroxyapatite is first detected.\textsuperscript{26} Calcification in the presence of MGP suggests that production may be “swamped” or that the MGP/Ca complex is unable to escape from the vessel wall, leading to accumulation of MGP protein.\textsuperscript{8} Alternatively, it is possible that in MS patients, MGP may be dysfunctional, because the levels of γ-carboxylase and its essential cofactor, vitamin K, are reduced in vessels with age.\textsuperscript{27} More studies are required to elucidate the precise role of MGP in human vascular calcification.

Expression of OP, an RGD-containing phosphoprotein that can bind calcium and mediate cell adhesion and migration, was high in macrophages in calcified atherosclerotic lesions but low in human medial VSMCs in normal and MS vessels.\textsuperscript{28} In a previous study,\textsuperscript{26} we showed that OP is not highly expressed by calcifying VSMCs in vitro; therefore, it is unlikely that OP promotes vascular calcification. Osteopontin is a powerful inhibitor of hydroxyapatite crystal formation in vitro.\textsuperscript{28} In the present study, despite low mRNA expression, the protein accumulated at the VSMC/calcification interface in a distribution pattern similar to that observed in bone and dentine, in which OP is thought to regulate the rate of mineralization and cement cellular and mineral junctions.\textsuperscript{29} The expression of OP by normal medial VSMCs and its accumulation in association with calcification suggest that OP may have a specific role as an inhibitor of calcification in the normal vasculature.

Expression of ALK, BSP, and BGP was significantly higher in MS than in normal vessels. ALK, an early marker of osteogenesis, is thought to promote mineralization by providing a source of orthophosphate for incorporation into CaPO$_4$ mineral.\textsuperscript{30} The function of BSP is less clearly defined, although in vitro evidence suggests it may be involved in the nucleation of hydroxyapatite at the mineralization front of bone.\textsuperscript{31} However, BSP also has an RGD-domain, which suggests that, like osteopontin, it may have roles in regulating mineralization and in promoting cell adhesion and migration.\textsuperscript{32} BGP, like MGP, contains calcium-binding Gla residues that can inhibit hydroxyapatite crystal growth in vitro.\textsuperscript{33} Mice lacking a functional BGP gene develop bones of higher density than normal, which suggests BGP is also a negative regulator of bone formation in vivo.\textsuperscript{34} However, the expres-
sion of BSP and BGP is not restricted to bone; both are expressed in soft tissues prone to calcification, which suggests that under specific conditions, they may be expressed by diverse cell types to regulate ectopic calcification.32,35

SPARC is an abundant matrix protein in bone, where it links collagen and mineral, whereas in vitro, it is one of the most potent inhibitors of hydroxyapatite crystal formation.30,36 The high expression of SPARC in the normal vasculature and its absence in MS lesions suggest that its loss may promote mineralization. In contrast, COL II was expressed at low levels in the normal vasculature but upregulated in MS lesions. COL II has been implicated in the regulation of mineralization, because it can bind annexin V, which is essential for matrix vesicle function.30 This is the first report of COL II expression in the human vasculature, and its association with medial calcification may be analogous to the “cartilaginous metaplasia” described in the MGP knockout mouse.

Are VSMCs Responsible for the Formation of Bone in MS?

BMP2 has previously been found in intimal calcification and can induce ectopic bone formation.10,37 However, its expression in both normal and MS vessels suggests it is unlikely to induce medial ossification. In MS lesions, there were no chondrocytes or osteoblasts, and the cells associated with calcification and bone were positive for α-SM actin and SM22α and therefore were likely to be VSMCs. Significantly, we found that noncloned, unpassaged human VSMCs exhibited an osteoblastic gene expression profile before they formed nodules and accumulated hydroxyapatite. Furthermore, this phenotype was common to VSMCs derived from normal vessels and MS lesions. Thus, VSMCs in vitro and in vivo can coexpress osteoblastic and VSMC-specific genes. However, it is unclear whether VSMCs can orchestrate bone formation, and it cannot be ruled out that other locally recruited cells, such as pericytes, or circulating cells with the potential to differentiate into osteoblasts may be responsible for the initiation of bone in MS lesions.38

Conclusions

The above observations imply MS is due to a regulated calcification process and that constitutive expression of MGP, OP, and SPARC in normal vessels inhibits calcification. However, in the presence of calcification, VSMCs express a number of osteocytic/chondrocytic markers that act to either facilitate or regulate the calcification process. The signals that initiate expression of osteocytic/chondrocytic proteins in human VSMCs remain to be determined. The spontaneous adoption of an osteocytic/chondrocytic phenotype by human VSMCs in culture may provide a model to test putative regulatory factors and potential therapeutic interventions to prevent vascular calcification.
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