Calcification of Human Vascular Cells In Vitro Is Correlated With High Levels of Matrix Gla Protein and Low Levels of Osteopontin Expression

Diane Proudfoot, Jeremy N. Skepper, Catherine M. Shanahan, Peter L. Weissberg

Abstract—The cellular and molecular events leading to calcification in atherosclerotic lesions are unknown. We and others have shown that bone-associated proteins, particularly matrix Gla protein (MGP) and osteopontin (OP), can be detected in atherosclerotic lesions, thus suggesting an active calcification process. In the present study, we aimed to determine whether human vascular smooth muscle cells (VSMCs) could calcify in vitro and to determine whether MGP and OP have a role in vascular calcification. We established that human aortic VSMCs and placental microvascular pericytes spontaneously form nodules in cell culture and induce calcification, as detected by von Kossa’s method, Alizarin red S staining, and electron microscopy. The cells in calcifying nodules differed from those in monolayer cultures by expressing higher levels of the SMC markers α-SM actin, SM22α, and calponin. In addition, Northern blot analysis revealed that in human VSMCs, calcification was associated with increased levels of MGP mRNA. In contrast, OP mRNA was barely detectable in calcified human VSMCs and pericyte nodules, nor was OP protein detected, suggesting that OP was not necessary for calcification to occur. These studies reveal that human VSMCs are capable of inducing calcification and that MGP may have a role in human vascular calcification. (Arterioscler Thromb Vasc Biol. 1998;18:379-388.)

Key Words: calcification ■ vascular smooth muscle ■ pericytes ■ osteopontin ■ matrix Gla protein

Calcification is a common and early event in the pathogenesis of atherosclerosis. Its presence is correlated with increased risk of myocardial infarction, and it predisposes to coronary dissection after angioplasty. Calcification also causes an increased brittleness and decreased elasticity of vessels, which may lead to other cardiovascular problems. The calcium deposits exist mainly in the form of hydroxyapatite, which is the type of calcium phosphate normally found in bone. However, the cellular and molecular events leading to calcification are largely unknown.

In a previous study in our laboratory, two genes coding for bone-associated proteins were identified, OP and MGP, which were preferentially expressed by proliferating rat VSMCs. In situ hybridization and immunohistochemical studies confirmed high expression of both proteins in human atherosclerosis, particularly associated with vascular calcification. Several studies have demonstrated other bone-associated proteins in calcified human atherosclerotic plaques, eg, OP, osteocalcin, bone morphogenetic protein 2a, collagen type I, and SPARC (osteonectin). In addition, structures such as matrix vesicles, which are the initial nucleation sites for hydroxyapatite mineral in bone, have been found in human atherosclerotic lesions.

The discovery of bone-associated structures and proteins in calcified human atherosclerotic lesions suggests an active calcification process that may be similar to that in bone formation. The cells responsible for this process in atherosclerosis are not known, but it is possible that vascular cells may be able to act in a manner similar to osteoblasts. It has been reported that bovine aortic SMCs, microvascular SM-like cells, and pericytes can differentiate in culture to form osteoblast-like cells and can produce a calcified matrix. Also, cloned human aortic cells with pericyte-like properties have been described.

In this study, we aimed to determine whether human vascular cells could calcify in vitro and if so, to investigate their pattern of gene expression during this process. We used VSMCs cultured from the medial layer of human aortas and made comparisons with pericytes isolated from microvessels of the placenta. We were particularly interested in the role of OP in human vascular calcification, since we previously found that human VSMCs express little OP when compared with rat VSMCs, yet OP protein is strongly associated with calcification in vivo. We also focused on the role of MGP in vitro calcification, since this protein was found in association with calcification in vivo.

Methods

Cell Culture
The culture medium used was M199 (GIBCO) or Dulbecco’s modified Eagle’s medium (Sigma Chemical Co) buffered with 3.7 g/L NaHCO₃ and 5% CO₂ and supplemented with 100 IU/mL penicillin...
**Selected Abbreviations and Acronyms**
- MGP = matrix Gla protein
- M199 = medium 199
- OP = osteopontin
- PCR = polymerase chain reaction
- VSMC = vascular smooth muscle cell

(Sigma), 100 μg/mL streptomycin (Sigma), 250 ng/mL amphotericin B (Sigma), and 4 mmol/L of L-glutamine (Sigma). Heat-inactivated fetal calf serum (FCS) was purchased from Sigma.

**Human VSMCs**
VSMCs were obtained from nonatherosclerotic areas of aortas from organ donors of various ages (males and females from 3 to 65 years old). The cells were prepared from explants of tissue and were confirmed as SM cells by positive staining with monoclonal antibodies against α-SM actin (A2547, Sigma). Cells were maintained in M199 containing 20% FCS and were used between passages 3 and 15. At least three different isolates from individuals of different ages were used in experiments.

**Human Placental Pericytes**
Samples of human placenta were collected within 1 hour of delivery. A central section of tissue rich in villi was dissected and washed thoroughly in serum-free M199. The tissue was then chopped into small pieces, and these pieces were placed in serum-free M199 containing 3 mg/mL collagenase (Sigma). The tissue was allowed to digest for 3 hours at 37°C in a shaking water bath. The digested material contained dispersed cells and small vessels. To separate the microvessels from the dispersed cells, the material was sieved through a membrane with a pore size of 70 μm (Falcon). Microvessels were carefully removed from the sieve and placed either into 25-cm² culture flasks (Falcon) or directly onto glass coverslips in 12-well plates, in M199 supplemented with 20% FCS. After 5 to 6 days in culture, pericytes as well as endothelial cells (confirmed by α-SM actin and von Willebrand factor immunohistochemistry, respectively) grew out from the microvessels. After two rounds of trypsinization, only α-SM actin–positive cells remained in these cultures. These cells were believed to be pericytes because of the following criteria: (1) they were isolated from microvessels; (2) their morphology was characteristic of pericytes; (3) postconfluent cells grew in multilayered nodules; and (4) they stained positively for α-SM actin. Pericytes were used between passages 2 and 5.

**Human Peripheral Blood Monocyte Isolation**
Human peripheral blood monocytes were isolated from preparations ofuffy coats purchased from the Blood Transfusion Service, Cambridge, UK. Mononuclear cells were isolated by sedimentation with Histopaque (Sigma). The monocytes were then separated from other leukocytes by adherence to plastic. The leukocyte suspension was plated out in 24-well plates (Falcon) at a density of 2×10⁶ cells per well, and nonadherent cells were removed after a 1-hour incubation at 37°C. Adherent monocytes were then washed three times with PBS and allowed to develop into macrophages by culturing in M199 supplemented with 20% FCS for 2 weeks. Human tissue was obtained and allowed to develop into macrophages by culturing in M199 containing 20% FCS and were used between passages 3 and 15. At least three different isolates from individuals of different ages were used in experiments.

**Detection of Calcification**
**von Kossa’s Method**
This method was adopted from Bancroft and Stevens. Cells were grown in 12-well plates, washed with PBS three times, and then fixed in 10% formaldehyde in PBS for 45 minutes at 4°C. The cells were washed with distilled water and exposed to 5% aqueous AgNO₃ and strong light for 60 minutes at room temperature. The cells were then exposed to 2.5% sodium thiosulfate for 5 minutes (black=positive staining). In some experiments, cells were further treated with Gieson’s picrofuchsin to visualize collagen (red=positive staining).

**Alizarin Red S**
Cells were prepared and fixed as detailed above. The cells were washed in distilled water and then exposed to 2% Alizarin red S (aqueous, Sigma) for 5 minutes (red/orange=positive staining).

**Electron Microscopy**
**Sample Preparation**
Nodules were fixed by immersion in either 2% glutaraldehyde or 4% formaldehyde in 0.1 mol/L PIPES buffer at pH 7.2 for 2 hours at 4°C. They were cryoprotected in 20% polypropylene glycol in 0.1 mol/L PIPES buffer at pH 7.2, subsequently frozen, and stored under liquid N₂. They were freeze-substituted in pure methanol, embedded in Lowicryl HM20, and polymerized by UV irradiation. Internal standards were prepared by dispersing hydroxyapatite crystals (Sigma) at a final concentration of 10% in 20% gelatin and embedding as described above.

Sections were cut on a Reichert-Jung ultratrace S microtome, mounted on glass slides, and stained with methylene blue to identify putative sites of calcification. Thin sections of these regions were examined by bright-field transmission electron microscopy to confirm the presence of putative calcification for subsequent elemental analysis.

**Scanning Transmission Electron Microscopy and Energy-Dispersive Microanalysis (STEM/EDX)**
Thin sections (150 to 200 nm) were cut on dry knives and mounted on 100-mesh Formvar/carbon/nickel grids. They were coated with 20 nm carbon; mounted in a copper/beryllium low-background, single-tilt holder; and examined in a Philips EM400. EDX was carried out in the STEM mode, and a solid-state, backscattered-electron detector was used to identify regions for EDX. This procedure was necessary because unstained, dry-mounted sections have little contrast even when examined by STEM. These areas were analyzed with a reduced raster for 150 seconds live time using an EDAX 9800 system. The detector efficiency for calcium and phosphorus was determined by using isotopic droplets and normalizing the counts for phosphorus against calcium to determine a correction factor for x-ray counts for phosphorus (79.75%). The ratio of calcium to phosphorus was determined by dividing the x-ray counts for calcium by the corrected x-ray counts for phosphorus. Fifteen analyses were carried out for each stage examined. Statistical comparison was made by ANOVA.

**Separation of Cells in Monolayer From Those in Nodules**
When monolayer cells were compared with cells in nodules, comparisons were made with cells from the same isolate, at the same passage, and grown as monolayer/nodules side by side. Monolayers were separated from nodules by trypsinization for <5 minutes. This process allowed dispersion of monolayer cells into a single-cell suspension while nodules retained their structure. Nodules were separated from single cells by passing them through a 70-μm filter (Falcon). In experiments where nodules were dispersed, nodules were incubated in 3 mg/mL collagenase (Sigma) at 37°C for 1 hour (longer incubation times lead to a decreased RNA yield). Cells dispersed from nodules retained the ability to re-form nodules if plated at a high density.

**RNA Isolation**
Cultured monolayer cells were trypsinized, and total cytoplasmic RNA was isolated by lysis in 150 mmol/mL NaCl, 10 mmol/mL Tris (pH 7.4), 1 mmol/L MgCl₂, and 0.5% Nonidet P-40 (Sigma). The nuclei were pelleted by centrifugation, and the supernatant was supplemented with 20% SDS to a final concentration of 1.5% and then extracted twice with Tris-buffered phenol. The RNA was precipitated with 10% (vol/vol) of 3 mol/L sodium acetate (pH 5.2) and 25% (vol/vol) ethanol, and the RNA pellet was resuspended in water. An identical procedure was used to isolate RNA from cells in nodules, except that nodules were dispersed for 1 hour in 3 mg/mL collagenase at 37°C before lysis. For isolation of RNA from placental
microvessels, the vessels were homogenized in 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.4), 1 mmol/L MgCl₂, and 1 μL/mL RNasin (ribonuclease inhibitor, Promega) before being treated with 0.5% Nonidet P-40 and following the above method. RNA from human aortic medial layers was prepared by cutting the media into small pieces and digesting them with 3 mg/mL collagenase and 0.5 mg/mL elastase (Sigma) until a single-cell suspension was obtained, followed by the above method.

Northern Blot Analysis

Total cytoplasmic RNA (10 to 15 μg per lane) was electrophoresed in 1.5% agarose gels containing 2.2 mol/L formaldehyde. The electrophoresis buffer contained 20 mmol/L MOPS, 1 mmol/L EDTA, 5 mmol/L sodium acetate, and 0.5 μg/ml ethidium bromide. The integrity of the RNA was visualized by UV illumination of the gels before and after transfer to Hybond-N (Amersham International). 32P-labeled cDNA probes were generated from purified insert DNA using an oligolabeling kit (Pharmacia). Filters were washed three times at 65°C (10-, 30-, and 60-minute washes) in 0.1X SSC/0.1% SDS before exposure to x-ray film (Fuji RX). The human OP and MGP probes were generated from full-length cDNA clones of 1.4 and 0.7 kb, respectively, that were obtained from the American Type Culture Collection/National Institutes of Health repository (Rockville, Md). The SM22α probe was generated from a 1.0-kb rat cDNA clone, 3RF10, and the α-SM actin probe was generated from a 1.4-kb rat cDNA clone, 3RD2.7

Reverse Transcription–PCR Analysis

RNA was prepared from VSMC and pericyte monolayers and nodules as described above. RNA from the osteoblast cell line SAOS (a gift from B. Nobel, Department of Medicine, Cambridge University) was included as a positive control. RNA (5 μg) was reverse-transcribed, and for PCR amplification, 2.5 μL of diluted cDNA was used in 20-μL reactions (as described in Reference 22). The cycling parameters were 94°C for 2 minutes, 55°C for 90 seconds, and extension at 72°C for 2 minutes for 30 cycles, with a final extension period of 6 minutes at 72°C. A 10-μL aliquot of each reaction was electrophoresed through a 1% (wt/vol) agarose gel, and the DNA was visualized by ethidium bromide staining under UV light transillumination. The oligonucleotide primers for human OP were 5′-ACTGATTTTCCACGGAGC-3′ (forward, nucleotides 466 to 483 of cDNA) and 5′-ATGAGCTGTTGAATCTCAGC-3′ (reverse, nucleotides 877 to 894 of cDNA) to give a predicted size product of 428 bp. Control primers to human β2-microglobulin, 5′-GATTCAGGTATTACGG-3′ (forward, nucleotides 51 to 68 of the cDNA sequence; exon 2) and 5′-CCATGATCTGCGTATCG-3′ (reverse, nucleotides 327 to 345 of the cDNA sequence; exon 3) were used to give a predicted product of 294 bp.22

Immunocytochemistry

Cells were plated on glass coverslips and allowed to develop into multicellular nodules. They were then washed three times with PBS, fixed in 3% formaldehyde for 45 minutes at 4°C, and permeabilized with 0.1% Nonidet P-40 before exposure to the primary antibody. Cells were exposed to blocking solution (5% BSA overnight at 4°C) and then to monoclonal antibodies recognizing α-SM actin (Sigma, diluted 1:200) and calponin (Sigma, diluted 1:200) for 1 hour at room temperature. Bound antibody was detected using anti-mouse conjugated FITC (Sigma, diluted 1:200). Controls were performed by substituting mouse serum for the primary antibody.

Western Blotting

Samples for protein analysis were prepared either from cell lysates or from cell-conditioned media. Cell lysates were prepared by washing the cells three times with PBS, removing the cells with a cell scraper, resuspending the cells in 0.25 mol/L Tris, pH 8.8, and freeze-thawing the resulting product three times (−70°C/37°C). The lystate was vortexed and then spun at 6000 rpm in a microfuge, and the supernatant was kept at −70°C until use. Nodular cell EDTA protein extracts were also prepared by using the method of Ramakrishnan et al.7 Conditioned media were collected in serum-free, phenol red-free Dulbecco’s modified Eagle’s medium (Sigma) for 48 hours. Samples were concentrated either by a factor of 10 by dialysis against 0.1X PBS and placed in Aquacide (Calbiochem) or by a factor of 100 by dialysis against 50 mmol/L NH₄HCO₃ and lyophilization. Samples were assayed for protein content by the Lowry method with a kit from Bio-Rad. Each sample of total protein (25 μg) was dissolved in sample buffer containing 100 mmol/L β-mercaptoethanol, boiled, and applied to a 10% polyacrylamide gel containing 0.1% SDS. Identical gels were either stained with silver (0.1% AgNO₃) or transferred to nitrocellulose (Immobilon-P, Millipore) by using an electrophotoblotting system (Bio-Rad). Western blot analysis was performed using MPP-110, a monoclonal antibody to rat bone OP, which recognizes human OP.3 This antibody was obtained from the Developmental Studies Hybridoma Bank in ppgl form and used at a dilution of 1:50. This antibody was detected with an anti-mouse horseradish peroxidase–conjugated antibody and chemiluminescense (ECL, Amersham).

Results

Human VSMCs and Microvascular Pericytes in Culture

VSMC were cultured from explants of human aortic media and confirmed as SMCs by expression of the SM cell marker α-SM actin. When the cells grew to confluence, they were either subcultured further or allowed to remain in confluent layers. Cells maintained in confluent culture formed a distinct “hill-and-valley” morphology, with cell retraction from some areas and coalescence into multicellular foci, or nodules, in other areas (Fig 1A). This phenomenon in human VSMCs has been reported previously.28–30 Pericytes were isolated from microvessels of human placental villi and confirmed as pericytes by expression of α-SM actin.21,31 The pericytes appeared morphologically distinct from human VSMCs, in that they were large, stellate cells with prominent actin fibers (Fig 1B; also shown in Fig 5B). Pericytes retracted and grouped into multicellular nodules (Fig 1B) in a manner similar to human VSMCs. Also, the perinodal cells in pericyte and VSMC cultures shared similar features (Fig 1). In cross section, nodular aggregates contained concentric layers of cells arranged around the outer part of the nodule, with many small cells in the middle of the nodule, a feature comparable to observations in VSMC nodules made by Bjorkrud and colleagues.32 It is well established that pericytes in nodular phenotype can stimulate calcification.12,32 We therefore investigated whether our nodular cultures of human VSMCs and pericytes were capable of calcification.

Calcification by Human VSMCs and Microvascular Pericytes in Culture

When the nodular cells were maintained for 30 days in culture, calcification was found to be associated with the nodules by Alizarin red S and von Kossa staining (Fig 2A through 2D). At this stage in culture, collagen was found to be associated with the nodular structures by staining with Gienson’s picrofuchsins (Fig 2E and 2F). To establish the chemical nature of the calcification, the nodular cells were analyzed by electron microscopy. Fig 3 demonstrates the presence of necrotic debris, highly electron-dense rounded structures (Fig 3A), and electron-dense elongated, needlelike structures (Fig 3B). Electron-dense structures were found in VSMCs and pericyte nodules. However, the needlelike structures were detected...
only in nodules with cell-free cores and were less frequent than the rounded structures. The rounded structures in Fig 3A are morphologically similar to matrix vesicles found in human aortic media, although the former were often larger than matrix vesicles found in bone. Both types of electron-dense areas were analyzed by EDX for elemental content and were found to consist of calcium and phosphorus in a ratio similar to that of hydroxyapatite (the Table). Human aortic SMCs and microvascular pericytes were therefore considered to be models for investigating the development of calcification in vitro.

Expression of SMC Markers
To characterize the cells in the nodular calcifying phenotype, expression of SM markers by these cells was compared with that of cells from the same culture in monolayer. By Northern blot analysis, it was found that α-SM actin and SM22α were both expressed at slightly higher levels in both human VSMC nodules and pericyte nodules compared with RNA isolated from cells in monolayer (Fig 4). In freshly dispersed aorta, SMC markers were poorly expressed. This may reflect a low turnover of the protein products in the mature vessel wall.

Both cell types in nodules expressed higher levels of α-SM actin protein, as demonstrated by immunocytochemical staining (Fig 5A and 5B). Cells dispersed from the nodules also expressed higher levels of α-SM actin (not shown). Human VSMC monolayer cells expressed this protein in a diffuse manner, but many of the pericytes contained prominent α-SM actin filaments within their cytoplasm (Fig 5A and 5B). Calponin protein was found to be associated almost exclusively with cells in nodules, with no staining in surrounding monolayer cells (Fig 5C and 5D). This observation was confirmed when nodular cells were dissociated by collagenase-dispersion and plated in fresh medium (Fig 5G and 5H). The nodular cells therefore retained the ability to express calponin when removed from the nodular environment.

Expression of MGP and OP
To determine whether calcification in the nodular cells was associated with bone-related proteins, expression of MGP and OP was investigated.

MGP Expression
Fig 6 shows that cells isolated from microvessels (containing pericytes and other cells contained within the microvessels) contained very little MGP mRNA. When pericytes were grown in culture, pericyte monolayer cells and nodular, calcifying cells expressed similar, higher levels of MGP mRNA. MGP levels were therefore unchanged when pericytes formed calcifying nodules. Human VSMC monolayer cells, however, expressed very low levels of MGP mRNA, but the cells in the nodular, calcifying phenotype expressed high levels of mRNA encoding MGP (Fig 6). MGP mRNA was also highly expressed by human VSMCs derived from freshly dispersed aortic medial SMCs (not cultured). These results show that pericytes and human VSMCs clearly differ in terms of MGP expression by nodular, calcifying cells and by cells freshly-dispersed from aorta or microvessels.

OP Expression
Cells isolated from placental microvessels showed high levels of OP expression (lane 3, Fig 6), as has been previously described, but medial VSMCs showed no OP expression (lane 6, Fig 6). The blot in Fig 6 contained VSMC RNA from a 3-year-old. In older individuals, OP mRNA was also not detected. Furthermore, whether in monolayer or calcifying nodules, neither VSMCs nor pericytes expressed OP mRNA detectable by Northern blot analysis (Fig 6). However, low levels of OP mRNA could be detected by PCR analysis in both monolayers and nodular cell cultures (Fig 7). These data are in agreement with a previous study in which we showed that OP mRNA could be detected in human cultured VSMCs only by PCR.
Since OP mRNA was detectable but did not appear to be upregulated in the calcifying nodular cells, we investigated whether OP protein accumulated within the nodular calcifying cells. Western blotting was performed using the monoclonal antibody MPIIIB101 raised against rat OP. Human placental microvessel lysate was used as a positive control (Fig 8), and MPIIIB101 also recognized OP protein in rat VSMC–conditioned and human macrophage–conditioned media (not shown). Using Ramakrishnan’s method for extracting OP from calcified nodules27 in a buffer containing 0.5 mmol/L EDTA, we did not detect OP protein in human calcified VSMCs or pericyte nodules (Fig 8A, lanes 5 and 8). To determine whether OP protein was actually produced in small amounts by human VSMCs and pericytes, conditioned media were prepared and concentrated by a factor of either 10 or 100. In the 10× concentrate, no OP was detected (data not shown), but in the 100× concentrate, OP was detected in conditioned media from monolayer and nodular cultures at the expected molecular weight of OP, ≈60 kDa (Fig 8A). The amount of OP present in VSMC- and pericyte-conditioned media was substantially lower than that found in placental microvessel extracts. We can therefore conclude that human VSMCs and pericytes secrete very low levels of OP protein but that OP does not accumulate in the nodular, calcifying cells.

Discussion
In this article, we have shown that VSMCs from human aorta and pericytes from human placenta formed multicellular nodules in postconfluent cultures and deposited a mineralized matrix after 30 days in culture. These findings are comparable with those from other studies with bovine VSMCs19 and pericytes21 and clones of human medial cells, which are believed to be pericyte-like.12 In our study, formation of nodules by human VSMCs and pericytes appeared to be a prerequisite for calcification. The rate of calcification in these nodular cells was somewhat slower than that observed in osteoblast cultures,34 where mineralized nodules appeared after 14 to 19 days in culture. However, bone cells in culture often require addition of cofactors, such as ascorbic acid, β-glycerophosphate, and dexamethasone, to the culture medium to induce calcification. In the present study, nodule formation and calcification occurred spontaneously without addition of exogenous factors. The factors responsible for inducing nodule formation and calcification are not known. Our results indicated that human vascular pericytes and VSMCs are capable of inducing calcification and that both cell types may therefore play a role in calcification in vivo.

Type of Calcification and Mechanism
Electron microscopic studies revealed that the elemental composition of calcified deposits in human vascular nodular cells was similar to that of hydroxyapatite, the type of calcium phosphate found in bone. Calcification in the nodules appeared as needle-like crystals or rounded structures, similar to the VSMC-derived matrix vesicles observed in calcified human aorta.15 In bone and cartilage formation, the initial site of calcification is thought to be associated with matrix vesicles that bud off from cell membranes of osteoblasts and chondro-
Calcifying Nodules

Expression of MGP by Cells in Calcifying Nodules

SM-associated genes such as α-SM actin, calponin, and SM22α are expressed at high levels in the medial layer of the vessel wall by adult, contractile VSMCs in vivo. These genes are not specific for VSMCs, but changes in the levels of expression of these genes are indicative of a particular VSMC phenotype. In atherosclerosis, VSMCs in the intima have a dedifferentiated, noncontractile phenotype and express lower amounts of the VSMC-associated genes.3,37 To investigate the phenotype of cells within calcifying nodules, genes expressed by these cells were compared with the same cell type grown in monolayer. Immunohistochemical staining revealed that human VSMCs and pericytes both expressed α-SM actin. However, α-SM actin staining was most intense in the cells around the outer layer of the nodules. Staining for calponin showed that this SMC marker was highly expressed only by cells in the nodular form. Both nodular pericytes and VSMCs also expressed higher levels of α-SM actin mRNA and SM22α mRNA than did monolayer cells. The types of vascular cells creating the environment for in vitro calcification are therefore different from the cells growing in monolayer in terms of their expression of VSMC markers. Expression of these particular markers indicates that the cells in the nodules appear to have a phenotype similar to that of VSMCs in adult contractile vessels, and that they “redifferentiate” in culture when they form nodules.

Expression of MGP by Cells in Calcifying Nodules

Further differences were noted between the nodular calcifying VSMCs and the monolayer cells when we investigated MGP mRNA expression. VSMCs in monolayer culture expressed

STEM revealed an abundance of necrotic cells within calcifying nodules. Indeed, significant amounts of hydroxyapatite were detected only in nodules with a necrotic core. Necrotic areas of human VSMCs have been demonstrated in other studies.32 This is comparable with investigations of desmoid calcification in vitro and in ossification of long bone cortices in fetal mice, where cell necrosis was found to be present during mineralization.35,36 In these studies, physiological cell death was thought to be a useful mechanism for the liberation of intracellular stores of calcium. Why cell death should occur in the central area of the nodule has not been determined. It is possible that cells in the core of the nodule do not receive enough nutrients from the culture medium, or perhaps cell death is caused by an orchestrated mechanism leading to calcification. In a study by Bjørkerud et al.,30 wherein human arterial VSMCs were studied by electron microscopy, many cells in the central core of nodules cultured for 1 week had features of apoptosis, ie, condensed chromatin and fragmented nuclei. Calcification was not observed in these early nodular cultures. Necrosis and apoptosis are certainly features of advanced human atherosclerotic plaques, and the bone-related proteins OP and MGP have been detected in association with necrotic lipid cores.8 Whether cell death by necrosis or apoptosis is involved or required in the calcification process is still to be elucidated.

Expression of SMC Genes by Cells in Calcifying Nodules

Ca/P Ratios for Native Hydroxyapatite Crystals, Hydroxyapatite Crystals Substituted Against Methanol (Used in the Preparation of Nodules), and Electron-Dense Areas of Nodules

<table>
<thead>
<tr>
<th>Sample/Substitution Medium</th>
<th>Ca/P Ratio</th>
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</thead>
<tbody>
<tr>
<td>Hydroxyapatite in gelatin</td>
<td>1.63±0.031</td>
</tr>
<tr>
<td>Pure methanol</td>
<td>1.67±0.034</td>
</tr>
<tr>
<td>Day 37 VSMC nodules</td>
<td>1.57±0.030</td>
</tr>
</tbody>
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EDX revealed that electron-dense areas of the nodules contained calcium and phosphorous in ratios which did not significantly differ from internal hydroxyapatite standards prepared in the same way (ANOVA, P<0.022). Values are means±SEM calculated from fifteen analyses per sample.
very little MGP, but nodular, calcifying cells expressed high levels of MGP mRNA. Similar observations have been reported in rat osteoblasts, in which calcifying cells expressed higher levels of MGP mRNA than did the noncalcifying phenotype. This dramatic upregulation of MGP was not observed in pericyte nodular calcifying cells. This observation implies that pericytes and VSMCs have different properties in culture and may have a different time course of induction of MGP in response to nodule formation or calcification. It was also interesting that MGP mRNA was reexpressed by VSMC nodular cells at high levels, but these levels were not as high as in freshly dispersed VSMCs from the aortic media. Together, these data provide evidence against the hypothesis that human calcifying VSMC nodules consist of pericyte-like cells, as described in other studies.12

MGP is a vitamin K–dependent bone-related protein that is a potent inhibitor of hydroxyapatite crystal formation in vitro, via γ-carboxylated Gla residues.39 MGP is expressed in bone and some soft tissues, including the aorta, and is thought to have a role in clearing excess calcium from tissues into the circulation, thus protecting against calcification.40,41 In atherosclerosis, MGP is believed to be trapped in the aorta by binding calcium in the form of hydroxyapatite and also by binding lipids.42 MGP trapped in atherosclerotic lesions would therefore be unable to clear excess calcium, and calcification would then be allowed to proceed. There is no direct evidence that MGP binds calcium in the vessel wall and that the calcium/MGP complex is then exported to the circulation. However, low levels of OP mRNA are expressed by human VSMCs, it was possible that significant amounts of protein could accumulate in the calcifying nodules. Using a monoclonal antibody against OP, we found that OP was secreted by human VSMCs and pericytes but was not detected in calcified nodules. Hence, although OP has been found in

Expression of OP by Cells in Calcifying Nodules
It had previously been suggested that OP might be involved in vascular calcification; therefore, expression of OP was also investigated. OP was originally isolated from bone and its functions, include binding to hydroxyapatite and attracting and binding osteoclasts to hydroxyapatite and collagen bridges.44 OP has been found in human atherosclerotic plaques, particularly associated with macrophages but also with SMCs in the same vicinity.45,46 OP appears to be associated with calcification and with necrotic lipid pools and has therefore been implicated in calcification in human atherosclerosis. In our in vitro studies, VSMCs and pericytes in monolayer or in nodules expressed very low levels of OP mRNA, detectable only by PCR. These results are consistent with our previous observations that unlike rat cells, cultured human VSMCs express barely detectable levels of OP mRNA.22 However, in other species such as bovine SMCs or pericytes, these cells have been reported to upregulate OP when in a calcifying, nodular phenotype.19,21 Our results may be due to culture conditions, but we have shown that OP is also expressed at levels undetectable in the media of healthy vessels by Northern blot analysis.

Given that low levels of OP mRNA are expressed by human VSMCs, it was possible that significant amounts of protein could accumulate in the calcifying nodules. Using a monoclonal antibody against OP, we found that OP was secreted by human VSMCs and pericytes but was not detected in calcified nodules. Hence, although OP has been found in
association with calcification in human atherosclerosis, our model of in vitro calcification has revealed that OP does not appear to be required in the initiation of calcification. In bone, OP can act as a glue between hydroxyapatite and cell matrix and is also thought to act as an inhibitor of mineralization. If OP does inhibit calcification, then perhaps a lack of OP allows calcification to proceed in the human vascular nodules. There is little doubt that OP is associated with calcification in atherosclerosis in vivo and that OP can be synthesized at low levels by VSMCs in vivo. However, the predominant source of OP in atherosclerosis appears to be the macrophage, and we can postulate from our in vivo and in vitro studies that OP sticks to the calcified deposits in atherosclerotic lesions rather than stimulating calcification.
In conclusion, human vascular cells can initiate calcification and in so doing, upregulate the expression of VSMC differentiation–associated genes. Calcification was not correlated with upregulation of OP mRNA levels, suggesting that OP may not be necessary for calcification to occur. However, there was an association between calcification and MGP expression, which requires further investigation.

Acknowledgments
This work was supported by a British Heart Foundation program grant. P.L.W. is a British Heart Foundation Professor, and C.M.S. is a British Heart Foundation lecturer.

References
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doi: 10.1161/01.ATV.18.3.379
_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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