Calcification of Vascular Smooth Muscle Cell Cultures Inhibition by Osteopontin

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Abstract—Calcification of vascular tissue is a common complication in aging, atherosclerosis, diabetes, renal failure, aortic stenosis, and prosthetic valve replacement. Osteopontin is a noncollagenous adhesive protein routinely found at sites of dystrophic calcification and synthesized at high levels by macrophages in calcified aortic valves and atherosclerotic plaques. In the present study, we have characterized the calcification of bovine aortic smooth muscle cell (BASMC) cultures in vitro and have studied the effects of exogenous osteopontin on mineral deposition. Induction of calcification in BASMC cultures was alkaline phosphatase–dependent and was characterized by a multilayer cell morphology. Mineral deposition occurred in the basal matrix of multilayered areas as indicated by von Kossa staining, and transmission electron microscopy and electron diffraction identified the mineral as apatite. Ultrastructural analysis of the cultures showed the presence of extracellular matrix vesicles, calcifying collagen fibrils, and nodular-type calcifications similar to those found in calcified heart valves and atherosclerotic plaques. Purified osteopontin (0.05 to 5 μg/mL) dose dependently inhibited calcification of BASMC cultures, whereas vitronectin and fibronectin had no effect. In contrast to the inhibitory mechanism of levamisole on mineral deposition, osteopontin did not inhibit alkaline phosphatase activity or reduce phosphorus levels in the culture medium. Addition of calcium to the cultures overcame the inhibitory effect of osteopontin on BASMC culture calcification and resulted in decreased levels of calcium in the culture medium and increased levels in the cell layer. Moreover, using high-resolution, colloidal-gold immunocytochemistry, osteopontin was found intimately associated with growing apatite crystals. These data indicate that the effect of osteopontin, although calcium-dependent, was not mediated by simple calcium chelation but most likely by direct interaction of osteopontin with crystal surfaces. These studies suggest that BASMCs can be used to model vascular calcification in vitro and that soluble osteopontin released near sites of vascular calcification may represent an adaptive mechanism aimed at preventing vascular calcification. *(Circ Res. 1999;84:166-178.)*

Key Words: osteopontin ■ vascular calcification ■ ultrastructure ■ smooth muscle cell ■ vascular disease

Dystrophic calcification is defined simply as mineralization that occurs in tissues other than bones and teeth in the absence of a systemic mineral imbalance.1 Dystrophic calcification is a common response to injury in many soft tissues and can have devastating consequences when it occurs in certain soft tissues including heart valves and arteries. It is also a common detrimental consequence of implantation of biomaterials, prostheses, and other medical devices. In the heart, valve calcification leads to mechanical dysfunction and ultimately to valve failure. In fact, dystrophic mineralization remains the leading cause of stenotic or regurgitant failure in native and bioprosthetic valves.2 In arteries, calcification is positively correlated with heart disease and increased risk of myocardial infarction,3 increased ischemic episodes in peripheral vascular disease,4 and increased risk of dissection after angioplasty.5 Importantly, the presence of calcification in arteries has recently been found to be predictive for death and myocardial infarction in high-risk, asymptomatic patients.6,7 The mechanisms regulating dystrophic calcification are not known. Recent morphological studies have shown that pathological calcification of blood vessels shares features with normal mineralization of bone and cartilage tissue. Matrix vesicles, postulated nucleation sites for formation of apatite mineral in cartilage, and perhaps bone, have been detected in calcified atherosclerotic lesions.8,9 Bone morphogenetic protein type 2, a potent osteogenic differentiation factor, also is expressed in vascular calcified lesions.10 Additionally, mineral-binding, gamma-carboxy-glutamate (Gla)–containing proteins such as osteocalcin and matrix Gla protein (MGP)11 have been localized to advanced atherosclerotic lesions.11–15 Furthermore, MGP-null mutant mice die within the first 2 months of age as a result of arterial rupture and heart failure due to extensive calcification of the large elastic and muscular arteries and the aortic valve.16

Aside from these molecules, we17–19 and others13,20–22 have reported that osteopontin is abundant at sites of calcification...
in human atherosclerotic plaques and in calcified aortic valves but is not found in normal arteries. Osteopontin is an acidic phosphoprotein normally found in mineralized tissues such as bones and teeth, as well as in kidney and epithelial linings of the body (see Reference 23 for review). In atherosclerotic plaques and calcifying aortic valves, osteopontin is highly localized to the surfaces of calcified deposits. The major source of osteopontin protein in both lesions is infiltrating macrophages that are intimately associated with the calcified deposits, although smooth muscle and endothelial cells also synthesize osteopontin but at much lower levels.17–19 These data suggest that osteopontin might be an important regulator of vascular calcification.

Although a prominent component of the extracellular matrix of bone, the function of osteopontin in hard tissue formation, mineralization, and turnover is not yet clear. The presence of osteopontin at calcification foci in the osteoid, its association with bone surfaces, and its ability to facilitate adhesion of osteoblast-like cells in vitro have suggested that osteopontin might function to promote bone formation.24 On the other hand, cell-free nucleation and crystal growth studies in metastable calcium phosphate solutions have suggested that osteopontin inhibits apatite formation.25,26 and interaction of osteopontin with osteoclast \( \alpha_\beta \) receptor has been shown to stimulate resorption of bone.27 To determine the potential role of osteopontin in vascular calcification, we have used an in vitro model wherein extracellular matrix produced by bovine aortic smooth muscle cells accumulates apatitic mineral after addition of the organic phosphate donor \( \beta \)-glycerophosphate.28 In the present study, we show that under these conditions, apatite is deposited along and within collagen fibrils in the presence of matrix vesicles, observations previously reported for calcified vascular tissues in vivo.8 Using this culture system, we have found that exogenously added osteopontin associates with apatitic crystals and is a potent inhibitor of vascular calcification by a mechanism that does not involve alkaline phosphatase inhibition or calcium chelation/sequestration but likely involves direct inhibition of apatite growth by binding to crystal surfaces. From these data, we hypothesize that osteopontin released by various cell types, including macrophages, at sites of vascular calcification may represent an adaptive mechanism aimed at preventing deleterious, ectopic mineralization.

Materials and Methods

Cell Culture

Bovine aortic smooth muscle cells (BASMCs) were obtained by a modification of the explant method originally described by Koss.29 Briefly, medial tissue was separated from segments of bovine thoracic aorta. Small pieces of tissue (1 to 2 mm) were loosened by a 1-hour incubation in DMEM containing 4.5 g/L glucose supplemented with 165 U/mL collagenase type I, 15 U/mL elastase type III, and 0.375 mg/mL soybean trypsin inhibitor at 37°C. Partially digested tissues were placed in 6-well plates and cultured for several weeks in DMEM containing 4.5 g/L glucose supplemented with 20% FBS at 37°C in a humidified atmosphere containing 5% CO\(_2\). Cells that had migrated from the explants were collected and maintained in growth medium (DMEM containing 15% FBS and 10 mmol/L sodium pyruvate supplemented with 100 U/mL of penicillin and 100 \( \mu \)g/mL of streptomycin). To confirm that the cells isolated from bovine aortic wall were vascular smooth muscle cells, \( \alpha \)-smooth muscle actin, vimentin, and calponin levels were examined by immunofluorescence as described below. All 3 antibodies stained >95% of the cells in a filamentous pattern (data not shown), indicating that the cells were of vascular smooth muscle origin. For all experiments, cells were used between passages 2 and 5.

Adhesive Proteins and Neutralizing Antibody

Rat osteopontin was purified from the conditioned medium of rat neonatal smooth muscle cell cultures as described previously.30 This preparation was judged to be >95% pure, on the basis of Coomassie staining and N-terminal sequence analysis. Rat plasma vitronectin (Sigma Immunochemicals) and bovine fibronectin (TELIOS Pharmaceutical Inc) were resuspended in PBS to a concentration of 0.5 mg/mL and stored frozen until use. Goat anti-\( \alpha \)-smooth osteopontin antibody OP-199 and nonimmune goat serum were prepared, and IgG fractions were purified as previously described.35

Histochemical Analysis

For immunofluorescence microscopy, BASMCs were cultured on 10-well heavy Teflon-coated microscope glass slides (Cel-Line Associates Inc) for 24 hours, fixed with cold methanol, blocked with PBS containing 2% BSA and 10% normal rabbit serum, and treated with monoclonal anti-\( \alpha \)-smooth muscle actin antibody (1A4, Sigma) and monoclonal anti-vimentin antibody (V9, Dako) diluted with PBS containing 2% BSA 1:50 and 1:25, respectively. Monoclonal anti-calponin antibody (CALP, a gift of Maria Frid, University of Colorado, Denver),31 was used without dilution. As a secondary antibody, FITC-conjugated rabbit anti-mouse IgG was used after dilution with PBS 1:30. Mouse nonimmune IgG was used as a control for the primary antibody. Greater than 95% of the BASMCs stained with all 3 muscle markers, proving their smooth muscle origin.

The expression of alkaline phosphatase was visualized by incubating citrate-acetone-formaldehyde-fixed cells at room temperature for 15 minutes with Naphthol AS-BI Alkaline Solution (Sigma). Mineral deposition was assessed by von Kossa staining (30 minutes, 5% silver nitrate), as previously described.32

Induction of Calcification

Calcification of BASMC cultures was induced by the method of Shioi et al.28 Briefly, BASMCs were cultured in growth medium for 4 days and then switched to calcification medium [calcification medium = DMEM (high glucose, 4.5 g/L) containing 15% FBS, 10 mmol/L sodium pyruvate, 10 \( \mu \)mol/L insulin, 50 \( \mu \)g/mL ascorbic acid, 100 U/mL penicillin, 100 \( \mu \)g/mL streptomycin, and \( \beta \)-glycerophosphate (1 \( \times \)10\(^{-5}\) mmol/L as indicated)] for 10 days. The medium was replaced with fresh medium twice a week. In the time course experiments, the beginning day of culture in calcification medium was defined as day 0. For immunodepletion studies, medium containing 10 \( \mu \)g/mL osteopontin was mixed with 20 mg/mL anti-osteopontin (OP-199) and incubated for 1 hour at room temperature. Two hundred fifty milligrams of protein A–Sepharose was added and incubated for 1 hour at room temperature. The antibody-protein A–Sepharose complexes were removed by centrifugation, and the remaining supernatant diluted 20-fold for use in calcification studies.

Quantitation of Calcium Deposition

Calcification was assessed by a modification of the method described by Jono et al.33 Briefly, the cultures were decalcified with 0.6 N HCl for 24 hours. The calcium content of the HCl supernatant was determined colorimetrically by the o-cresolphthalein complexone method (Calcium Kit, Sigma). After decalcification, the cultures were washed with PBS and solubilized with 0.1 N NaOH/1.1% SDS. Total protein content was measured with a Bio-Rad protein assay kit (Bio-Rad). The calcium content of the cell layer was normalized to protein content.
Alkaline Phosphatase, Phosphorus, and Calcium Ion Assays

For cellular alkaline phosphatase activity measurements, cells were washed 3 times with PBS, and cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for alkaline phosphatase activity on the basis of the method of Bessey et al.34 One unit was defined as the activity producing 1 nmol of p-nitropheno1 within 1 minute. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad). The data were normalized to the protein content of the cell layer.

Phosphorus and calcium concentrations in the culture medium were measured by the phosphomolybdate complex method (Phosphorus Kit, Sigma) and the o-cresolphthalein complexone method (Calcium Kit, Sigma), respectively.

Ultrastructure Analysis and Mineral Characterization

For ultrastructural examination of BASMC cultures by transmission electron microscopy, cells grown on plastic were fixed overnight in an aldehyde solution containing 1% glutaraldehyde and 1% paraformaldehyde buffered with 0.1 mol/L sodium cacodylate buffer at pH 7.2. The cultures were then washed with 0.1 mol/L sodium cacodylate buffer alone, dehydrated in a graded series of ethanol solutions, and infiltrated and embedded in either Taab epoxy resin or LR White acrylic resin (Marivac). The resins were polymerized for 2 days at 55°C. Samples destined for epoxy embedding were also post-fixed with potassium ferrocyanide–reduced 4% osmium tetroxide to provide additional membrane contrast in the electron microscope. For mineral analyses by selected-area electron diffraction, other cultures were treated nonaqueously by fixing only with 100% ethanol, followed by direct embedding in resin without further processing. One micrometer-thick survey sections were prepared from various regions of the cultures and stained with Toluindine blue for examination by light microscopy. Thin sections (80 to 100 nm) of selected regions were then cut using a diamond knife on a Reichert Ultracut E microtome and placed on 5% dichloroethane–coated nickel grids evaporated with carbon. Grid-mounted sections were stained briefly with ethanolic uranyl acetate and lead citrate and examined using a JEOL JEM 1200EX transmission electron microscope operating at 60 kV. Anhydrously treated samples left unstained were used for selected-area electron diffraction using a 100 nmol/L diffraction aperture and a camera length of 80 cm. Diffraction patterns were analyzed and compared with synthetic apatite standards and powder diffraction files as previously reported for bone mineral.35

Osteopontin Immunogold Labeling

BASMCs were cultured in calcification medium for 7 days to allow mineralization to begin. Purified rat osteopontin (0.5 μg/mL) was then added until day 10. Cultures were preserved as described above using aldehyde fixative followed by embedding in LR White acrylic resin for immunocytochemistry. Postembedding immunolabeling was performed using osteopontin antibody (OP-199) and protein A–colloidal gold complex as described previously.36 Briefly, thin (80-nm) sections of the cultures were placed on nickel grids and incubated for 5 minutes with 1% ovalbumin in PBS, followed by incubation with primary antibody for 1 hour, rinsing with PBS, blocking again with ovalbumin, and then exposure to protein A–gold complex for 30 minutes. After final rinsing with distilled water, grids were air-dried and conventionally stained with uranyl acetate and lead citrate and viewed by transmission electron microscopy. The specificity of the OP-199 antibody has been shown previously by Western blotting35 and by incubations performed in the present study with use of preimmune serum and protein A–gold complex alone.

Statistical Analysis

Unpaired Student t test was used to compare groups. A value of P<0.05 was considered significant.

**Results**

β-Glycerophosphate Dose Dependently Induces Mineral Deposition in BASMC Cultures

As shown in Figure 1a, BASMCs cultured on tissue culture polystyrene initiated calcium-containing mineral deposition in a time-dependent manner when treated with calcification medium containing β-glycerophosphate. In contrast, BASMCs cultured in growth medium that lacked β-glycerophosphate did not calcify. As shown in Figure 1b, addition of β-glycerophosphate resulted in an increased phosphorus concentration in the BASMC culture medium, which correlated positively with calcium deposition in the cell layer. Conversely, calcium concentration decreased in the culture medium as the cell layer became calcified (Figure 1c). These findings are similar to and confirm those reported by Shioi et al.28 Interestingly, mineralization of the cultures was not detectable before day 7, even though phosphorus levels in the media were elevated even at early times.
increasing concentrations of phosphorus concentration in the culture medium increased with the initial concentration of medium were dose-dependent. Calcium deposition depended on phosphorus concentration, and calcium concentration in the medium was inversely proportional to calcium (c) concentrations in BASMC cultures. Calcium and phosphorus levels were measured at day 10 by the o-cresolphthalein complexone method, respectively. For calcium deposition measurements (a), the calcium content in the cell extract was normalized to cellular protein content. Data are presented as mean±SEM; n=3.

The effects of β-glycerophosphate on calcium deposition, phosphorus concentration, and calcium concentration in the medium were dose-dependent. Calcium deposition depended on the initial concentration of β-glycerophosphate (Figure 2a), and was half-maximal at ~4 mmol/L β-glycerophosphate. Phosphorus concentration in the culture medium increased with increasing concentrations of β-glycerophosphate (Figure 2b). This is most likely due to the action of alkaline phosphatase in liberating inorganic phosphate from the organic phosphate donor β-glycerophosphate (see below). Calcium concentration in the culture medium was inversely proportional to calcium deposition in the cell layer, as expected (Figure 2c). The calcification was not due to spontaneous precipitation of mineral from the media, because culture medium supplemented with up to 10 mmol/L inorganic phosphate failed to form calcified deposits in the absence of cells (data not shown). Furthermore, addition of calcification medium containing 10 mmol/L β-glycerophosphate to bovine aortic endothelial cell or mouse 3T3 cultures failed to induce mineralization (not shown). Thus, the calcification of BASMCs under conditions that elevate inorganic phosphate in the media is most likely to be a specific cell- and matrix-mediated event (also, see below). It should be noted that although culture conditions in the present study contained calcium concentrations in the normal physiological range (1.8 to 2.3 mmol/L), the phosphorus levels achieved after addition of 4 to 10 mmol/L β-glycerophosphate were much higher than those observed in normal adult serum (range, 1.2 to 2.4 mmol/L).

Figure 2. Effects of β-glycerophosphate (β-GP) on calcium deposition in the cell layer (a) and medium phosphorus (b) and calcium (c) concentrations in BASMC cultures. Calcium and phosphorus levels were measured at day 10 by the o-cresolphthalein complexone method and the phosphomolybdate complex method, respectively. For calcium deposition measurements (a), the calcium content in the cell extract was normalized to cellular protein content. Data are presented as mean±SEM; n=3.

To further determine whether the calcification process in BASMC cultures represented a physiological type of mineralization, histochemical, ultrastructural, and electron diffraction analyses were performed. By light microscopy, BASMC cultures grown in growth medium showed areas of monolayer and multilayered growth typical for these cells (data not shown). After treatment with calcification medium for 10 days, the cultures showed most extensive deposition of mineral predominantly in multilayered areas (Figure 3a). Von Kossa staining confirmed the presence of phosphate-containing mineral in these cultures (Figure 3c and 3d). The calcification was most often observed in the extracellular matrix between cells and was typically more pronounced at the basal aspect of the culture (Figure 3d). The BASMCs in these calcified cultures were also positive for alkaline phosphatase activity, as shown in Figure 3b.

At 14 days of culture (10 days with calcification medium containing 10 mmol/L β-glycerophosphate), BASMCs (Figure 4a through 4c) were monolayered or multilayered and at some locations formed nodules of cells. Ultrastructurally, where multilayered or nodular in appearance, the cells were associated with abundant extracellular matrix rich in collagen fibrils. At sites of this extracellular matrix accumulation, cells exhibited well-developed organelles typically associated with protein synthesis and secretion (Figure 4b) and a prominent cytoskeleton as evidenced by an extensive network of intra- and intercellular microfilaments (Figure 4c), most likely composed principally of actin. Whereas cells cultured without β-glycerophosphate showed no evidence of extracellular matrix calcification, those cultured with the added organic phosphate source showed several morphologically distinct forms of calcification associated with the cell layer (Figure 5a through 5c). These included roughly spherical aggregates of calcified collagen fibrils (Figure 5a), nodular deposits with increased mineral density at the periphery (Figure 5b), and more diffuse calcification involving both the intra- and interfibrillar compartments of the extracellular matrix (Figure 5c). At these latter sites, crystals having somewhat larger dimensions were observed to extend from one collagen fibril to another. Membrane-bounded matrix vesicles were also found in the extracellular matrix (Figure 5d). Selected-area electron diffraction of anhydrously treated and unstained tissue sections of BASMC cultures containing calcified deposits identified the mineral phase as apatite, showing prominent diffraction reflections (from lattice planes 002, 211, 112, and 300) whose indices were characteristic for this type of mineral (Figure 5e).

Morphology of BASMC Culture Calcification

Inhibition of BASMC Culture Calcification:

Effects of Levamisole and Osteopontin

Alkaline phosphatase is required for normal bone mineralization and has been shown previously to be required for...
calcification of osteoblast and cartilage cell cultures in response to β-glycerophosphate. To determine whether alkaline phosphatase was required for calcification in BASMCs under the conditions used in the present study, we treated cultures with the alkaline phosphatase inhibitor levamisole or with vehicle alone. As shown in Figure 6a, calcium deposition in BASMC cultures was dose dependently inhibited by levamisole. Half-maximal inhibition was observed at 5 × 10^−5 mol/L levamisole. Vehicle treatment alone had no effect (data not shown). As expected, levamisole treatment was associated with a decrease in phosphorous concentration and maintenance of high calcium concentrations in the culture medium (Figure 6b and 6c).

Osteopontin is abundantly secreted by macrophages infiltrating both calcified aortic valves and atherosclerotic plaques and is typically found associated with the mineral deposits found in these lesions, suggesting that it may be involved in regulating dystrophic calcification. To examine the effect of osteopontin on BASMC-mediated calcification in vitro, we applied soluble osteopontin or vehicle alone (0.1 mmol/L sodium citrate) to the calcifying BASMC cultures. As shown in Figure 7a, osteopontin at 0.05, 0.5, and 5 μg/mL dose dependently inhibited calcification assessed at 10 days. In contrast, vehicle alone had no effect.

To exclude the possibility that contaminants in the osteopontin preparation might be responsible for the inhibitory effect, we immunodepleted medium containing 0.5 μg/mL rat osteopontin antibody (OP-199) or normal goat IgG. Before immunodepletion, this osteopontin solution inhibited calcification of the cultures by 18-fold (5.05 ± 0.25 μmol/mg for vehicle-treated versus 0.33 ± 0.06 μmol/mg for osteopontin-treated BASMCs, P = 0.0023). Immunodepletion of the osteopontin solution with osteopontin antibody significantly reduced its inhibitory activity (0.33 ± 0.06 μmol/mg for nonimmunodepleted sample versus 2.60 ± 0.43 μmol/mg for anti-osteopontin-depleted samples, P = 0.0338). In contrast, immunodepletion with normal goat IgG did not affect the inhibitory activity of the rat osteopontin solution (0.49 ± 0.10 μmol/mg with no immunodepletion, P = 0.2480).

To exclude the possibility that osteopontin was cytotoxic, we examined the effect of osteopontin washout on BASMC mineralization. Osteopontin was added to BASMCs on day 0 and cells were allowed to incubate for 3 days. After this time, the media was removed, cells washed, and fresh calcification medium containing 10 mmol/L β-glycerophosphate was added. Calcium deposition was then measured at day 7. No significant difference in mineralization was observed when compared with control cultures that were incubated with calcification medium containing 10 mmol/L β-glycerophosphate alone for the entire 7 days (osteopontin-treated = 8.5 ± 0.5 μmol/mg protein versus 8.0 ± 0.25 μmol/mg protein, respectively, P > 0.05).

To determine the specificity and uniqueness of the effects of osteopontin, we tested 2 additional noncollagenous extracellular matrix molecules that share limited structural and functional homology with osteopontin: vitronectin and fibronectin. Interestingly, at equimolar concentrations, neither protein inhibited BASMC-mediated calcification (Figure 7b).

**Figure 3.** Morphology (a through c) and alkaline phosphatase activity (d) of BASMCs. a, By light microscopy, opaque areas were observed about 10 days after switching to calcification medium (×40 magnification). b, Von Kossa staining (black) shows diffusive and granular calcification predominantly in the extracellular matrix regions of the cultures. Cells were counterstained with hematoxylin (×100 magnification). c, von Kossa staining of a cross section of the BASMC cultures showing basal deposition of mineral (black) between multilayered cells. Cells were counterstained with hematoxylin (×100 magnification). d, BASMCs stained for alkaline phosphatase activity (violet). Cells were counterstained with hematoxylin (×200 magnification).
Thus, the effect of osteopontin under these conditions was highly specific.

**Mechanism of Osteopontin-Induced Inhibition of Calcification**

We next examined the mechanism by which osteopontin inhibited calcification of the BASMC cultures. We initially considered 2 possibilities based on the characteristics of the model system and on the properties of osteopontin. First, osteopontin might function in a manner similar to levamisole by affecting alkaline phosphatase activity, thereby inhibiting production of inorganic phosphate from γ-glycerophosphate and preventing calcium phosphate deposition. However, as shown in Figure 8a and 8c, alkaline phosphatase activity of BASMCs was not effected by treatment with osteopontin. Likewise, the phosphorus content of the medium was not decreased by addition of osteopontin (Figure 8b). In contrast, levamisole dose dependently inhibited BASMC alkaline phosphatase activity (Figure 8c) and reduced the phosphorus concentration in the culture medium as expected (Figure 6b). Thus, osteopontin does not act by inhibiting alkaline phosphatase activity.

A second possibility is that osteopontin might act to chelate or sequester calcium in the culture medium, thereby preventing mineralization. To test this, we supplemented the initial calcification medium with increasing concentrations of calcium in the presence of osteopontin or vehicle alone. Cultures were then allowed to calcify as usual in the presence or absence of osteopontin over a 10-day period. As shown in Figure 9a, increasing the calcium content of the medium was able to overcome the inhibitory effect of osteopontin on calcium deposition, allowing more mineral to be deposited in the cell layer. Consistent with this, a decrease in the phosphorus content (from 8.2 mmol/L to 7.3 mmol/L) of the culture medium was noted (Figure 9b).

We next measured the calcium content of the medium at the end of the 10-day period in the presence of osteopontin. If the inhibitory effect of osteopontin was due to calcium sequestration, we expected to see either a constant or increasing amount of calcium in the culture medium, reflecting retention of calcium in the medium by osteopontin binding. However, the opposite was observed. Calcium concentration in the culture medium was decreased at the end of the 10-day period compared with initial calcium concentrations and...
Figure 5. Electron micrographs selected to show various calcification (Cal) events associated with the collagenous (Coll) extracellular matrix produced by BASMCs (SMCs) after 10 days in calcification medium. a, Electron-dense calcified deposits frequently appear as discrete, micrometer-sized masses within the collagen-rich extracellular matrix surrounding the cells. Closer examination of the calcification pattern reveals that at the margins of these sites, a finely textured mineral commonly aligns with fibrillar collagen and progresses along individual collagen fibrils (arrowheads, inset). Additional mineral deposition appears to occur within the interior of these calcification foci, extending into the interfibrillar volume as well, thus rendering them more electron dense and homogeneous. Nu indicates nucleus; m, mitochondria. b, Another calcification pattern typically seen in the extracellular matrix of the BASMC cultures consists of small nodular accretions of mineral (asterisks) with an increased density at their periphery (open arrows). c, Frequently observed at the margins of calcification sites are relatively large crystals (small arrows) spanning the interfibrillar compartment and passing from one collagen fibril to another. This mineral texture differs from that seen in panel a (inset). d, Membrane-bounded matrix vesicles (mv) are occasionally encountered within the extracellular matrix. e, Selected-area electron diffraction for mineral characterization from an unstained LR White section of an anhydrously prepared sample identifies predominantly apatite within the calcified deposits, with diffraction maxima typical for apatite as indicated.
correlated inversely with calcium deposition (compare Figure 9a and 9c). Thus, although the inhibitory effect of osteopontin on mineralization was calcium-dependent (ie, decreased by increasing calcium concentrations), it does not appear to be simply attributable to chelation of the calcium available in the medium.

Finally, we examined the ultrastructural localization of endogenous and exogenous osteopontin in the BASMC cultures by immunogold labeling. For these experiments, osteopontin was omitted (vehicle alone) or added on day 7 after initiation of mineralization with calcification medium containing 10 mmol/L β-glycerophosphate. Under these conditions, exogenously applied osteopontin (0.5 μg/mL) was still able to inhibit BASMC culture calcification by 50% at day 10 (data not shown). As shown in Figure 10a, a low level of endogenous osteopontin was found in untreated, mineralizing cultures, typically in a diffuse pattern in the mineralized areas. In contrast, in osteopontin-treated cultures, gold particles were abundant at sites of calcification, typically accumulating at the margins of small calcified masses (Figure 10b) or associating with individual crystal profiles (Figure 10c). No gold particles were observed when preimmune serum and protein A–gold complex alone were used as controls (data not shown). These data suggest that a direct interaction of osteopontin with the growing apatite crystals is required for its inhibitory function.

Discussion
Calcification of vascular tissue is a common finding in atherosclerosis, diabetes, renal failure, aging, aortic stenosis, and prosthetic valve replacements. Osteopontin, an acidic noncollagenous protein, is invariably found at sites of dystrophic calcification.13,14,17–22 To investigate the mechanisms of vascular calcification and osteopontin function in this context, we have examined the mineralization of vascular smooth muscle cell cultures in vitro. Our findings indicate that calcification of the matrix deposited by BASMCs resembles the mineralization observed in atherosclerotic plaques and other types of dystrophic calcification with regard to (1) mineral type (apatite), (2) the association of mineral with extracellular collagen fibrils, and (3) the presence of matrix vesicles. Furthermore, osteopontin profoundly inhibits the mineralization of these cultures by a mechanism distinct from calcium chelation or inhibition of alkaline phosphatase activity but most likely involves direct adsorption and inhibition of apatite crystal growth.

Our studies support and extend previous findings of Shioi et al28 using BASMCs treated with β-glycerophosphate as a
model for vascular calcification. In agreement with that work, we found that alkaline phosphatase activity was required for BASMC-mediated calcification in the presence of β-glycerophosphate. However, in the previous reports, the type of mineral formed, the presence of extracellular matrix, and the ultrastructure of the calcified deposits and their matrix relationships were not determined, leading to questions regarding the physiological relevance of the observed calcification. In the present study, we show that apatite is the predominant mineral formed in BASMC cultures under these conditions and that the mineral deposits share considerable morphological similarity to deposits found in calcifying atherosclerotic plaques as well as bone, cartilage, and teeth.8,9 First, mineralization of the BASMC cultures occurred predominantly extracellularly in association with collagen fibrils and rarely intracellularly or associated with necrotic debris. Second, nodular calcifications suggesting spherulitic crystal growth, which are commonly observed in calcified atherosclerotic plaques and valves,8 were also present in calcifying BASMC cultures. Finally, we identified matrix vesicles in association with calcifying extracellular matrix in the BASMC cultures. These vesicular structures have been reported in calcified atherosclerotic plaques in association with elevated alkaline phosphatase activity,8 suggesting that they may play a role in vascular dystrophic calcification as postulated for bone, calcified cartilage, and teeth.39 Moreover, matrix vesicles were also observed in the calcified arterial media of the MGP-null mice.16 Thus, the BASMCs used in the present studies are able to create an extracellular milieu capable of mineralization that, in many ways, is morphologically similar to that observed in calcified vascular tissues in vivo.

Apatite formation has also been demonstrated in pericytes derived from retinal microvessels40 as well as cloned bovine aortic medial cells, termed calcifying vascular cells,10 when grown in culture. In the former cells, the
Ultrastructure of mineral deposition was quite similar to that observed in the β-glycerophosphate–treated BASMCs used in the present study, including presence of calcifying collagen fibrils, matrix vesicles, and agglomerated calcifications. In contrast to the BASMCs used in the present study, however, pericytes and calcifying vascular cells were able to mineralize their matrices spontaneously in the absence of an exogenous phosphate donor. Interestingly, addition of β-glycerophosphate or culturing on a collagen substrate substantially accelerated calcification of these cultures. The ability of pericytes to induce calcification in the absence of an exogenous phosphate donor may be related to their ability to form multicellular nodules and thereby create calcium- and phosphate-rich microenvironments. However, we have been unsuccessful in eliminating the β-glycerophosphate requirement of BASMCs by culturing on a glass substrate, which enhances nodule formation in these cultures substantially (Wada and Giachelli, unpublished observation, 1996). Alternatively, the requirement for exogenous β-glycerophosphate may be related to as yet unknown mineralization promoting factors absent (or conversely, mineralization-inhibitory factors present) in the various types of culture medium or secreted by the cells themselves. In support of this, lipid oxidation products, β-estradiol, transforming growth factor-β, and thrombospondin-1 have all been shown to modulate mineralization of pericyte cultures, and parathyroid hormone–related protein dramatically inhibits calcification of β-glycerophosphate–treated BASMCs. It is also notable that we have been able to observe spontaneous calcification in cultures of human aorta–derived smooth muscle cell under conditions of physiological calcium and phosphorus levels (Jono and Giachelli, unpublished observation, 1997). Clearly, a deeper understanding of the requirements for calcification by particular vascular cells is needed to address these issues.

Noncollagenous bone proteins including secreted, acidic phosphoproteins and Gla-containing proteins have been suggested to regulate physiological mineralization in general and have recently been associated with dystrophic calcification as well. Transgenic mice lacking MGP have recently been generated and show extensive dystrophic calcification of muscular and elastic arteries (in addition to premature and excessive cartilage calcification), suggesting that this protein may normally serve to inhibit the calcification process in the vasculature. In addition, osteocalcin, osteopontin, and osteonectin have also been found in calcified natural and prosthetic aortic valves, as well as calcified atherosclerotic lesions, suggesting a role for these proteins at these dystrophic calcification sites as well. Although studied most extensively in bone, the precise functions of these proteins are still not understood.

Figure 10. Localization of endogenous and exogenous osteopontin in mineralizing BASMC cultures. a, After immunocytochemical labeling for endogenous osteopontin, gold particles are diffusely located over calcified areas (CAL) of the extracellular matrix. Collagen fibrils at the periphery of such areas appear calcified (arrowheads). b, After addition of osteopontin to the cultures, calcified masses are somewhat smaller and exhibit an immunolabeling pattern distinct from that of endogenous osteopontin alone in that intense immunolabeling is observed at the margins of the calcified deposits (asterisks, CAL) and in association with peripherally located, calcified collagen fibrils (arrowheads). Coll indicates uncalcified collagen fibrils. c, At other calcification sites (CAL) within the cultures, osteopontin is intimately associated with relatively large crystal profiles (arrows) found among the collagen fibrils.
We have been particularly interested in elucidating the function of osteopontin because of its consistent association with calcified vascular tissues in vivo. Although the role of osteopontin in physiological mineralization has been controversial, in bone, osteopontin has been proposed to act as a regulator of bone formation by inhibiting calcification. Osteopontin is present in the osteoid matrix at small calcification foci, while levels of osteopontin increase dramatically at the mineralization front deeper in the bone. In other studies, osteopontin has been shown to facilitate the adhesion of osteoblast-lineage cells, particularly osteocytes and bone-lining cells, both of which suggest a role in bone-formative processes. However, osteopontin also promotes osteoclast resorption of bone, thus alternatively suggesting a degradative role.

In the present study, osteopontin was found to profoundly inhibit BASMC matrix mineralization at very low concentrations. To the best of our knowledge, this is the first study to test the effect of exogenously applied osteopontin on extracellular matrix mineralization in a calcifying cell culture system. The mechanism of this inhibition appears to be distinct from that of levamisole, an alkaline phosphatase inhibitor that inhibits the release of phosphate and also blocked calcification in this model. Thus, osteopontin treatment of BASMC cultures did not reduce alkaline phosphatase activity or phosphorus levels in the culture medium. We also ruled out simple calcium chelation/sequestration by osteopontin, because we did not observe retention of calcium in the medium by osteopontin at any time. Thus, although the inhibitory effect of osteopontin on mineralization was calcium-dependent (ie, decreasing with increasing initial calcium concentrations), it did not appear to be simply due to chelation of free calcium available in the medium. This is also consistent with the calcium-binding properties of osteopontin, which have shown that ~50 molecules of calcium can be bound by osteopontin at physiological calcium concentrations. Hence, it would require roughly 40 \( \mu \text{mol/L} \) osteopontin (2.7 mg/mL) to chelate 2 mmol/L calcium, which is more than 5000 times the amount of osteopontin used (0.5 \( \mu \text{g/mL} \)) and demonstrated to be effective in inhibiting vascular calcification in our assay. The ability of calcium to overcome the effect of osteopontin may be related to a calcium-sensitive conformational change in osteopontin, which has been previously described.

Our studies strongly suggest that osteopontin inhibition of mineralization occurs via direct binding of this protein to crystal surfaces. In osteopontin-treated cultures, osteopontin was found to be intimately associated with apatite crystals and not with unmineralized matrix or cells. Consistent with this hypothesis, osteopontin was able to inhibit mineralization even when added after nucleation was allowed to proceed, suggesting that it can bind to growing crystal surfaces and inhibit any further propagation. Likewise, the accumulation of osteopontin (as laminae limitantes) on bone surfaces as well as in the concentric organic lamellae of kidney stones, where growth and calcification have been arrested in vivo, supports this hypothesis. Our findings are also consistent with in vitro studies of urinary osteopontin, which was found to inhibit calcium oxalate precipitation, and cell-free nucleation studies in which osteopontin failed to nucleate hydroxyapatite crystals but potently inhibited crystal growth in both gelatin and agar gels. Taken together, these studies support the concept that an important function for osteopontin in mineralizing tissues is as an inhibitor of crystal growth during calcification.

Osteopontin may play multiple roles in the vascular response to injury. We and others have observed that osteopontin can facilitate adhesion and migration of vascular smooth muscle and endothelial cells and increase the survival of endothelial cells. These processes are integrin-mediated and require the arginine-glycine-aspartate (RGD) domain of osteopontin. These functions of osteopontin may be particularly important for early phases of tissue remodeling such as in arterial neointimal formation and angiogenesis, in which osteopontin and its receptors are expressed simultaneously by migrating vascular cells and might facilitate autocrine adhesion, migration, and/or survival of these cell types. In fact, inhibiting osteopontin with neutralizing antibodies was found to block arterial intimal formation after balloon catheter injury in the rat.

The ability of osteopontin to regulate vascular matrix mineralization may be more important at later phases of tissue remodeling in response to injury. In atherosclerotic plaques and calcifying aortic valves, osteopontin is intimately associated with calcified deposits. In both pathologies, macrophages associated with the chronic inflammation seen in these diseases appear to be the major source of osteopontin. Our findings suggest that the high levels of osteopontin secreted by macrophages may be a protective mechanism aimed at preventing pervasive vascular calcification. Although there is no evidence that osteoclast-like resorption of mineral deposits occurs in calcified vascular tissues, the finding that osteopontin might act as an opsonin for apatite-containing particulate phagocytosis by macrophages during wound healing is also consistent with an anti-calcific role of osteopontin in vascular calcification.

The capacity of osteopontin to modulate mineralization may be unrelated to its cell RGD-dependent adhesive functions. Calcium-binding domains in osteopontin have been identified and include an aspartate-rich domain and phosphorylated residues. Our finding that vitronectin and fibronectin, both RGD-containing peptides, failed to inhibit mineralization emphasizes that non-RGD sites in osteopontin are important in regulating mineralization. In support of this, preliminary data indicate that RGD peptides do not affect mineralization nor do they block the inhibitory effect of osteopontin on calcification of BASMC cultures (Wada and Giachelli, unpublished observation, 1996). Thus, osteopontin appears to exist as a multifunctional protein whose diverse sequence motifs operate not only at the cellular level but in the extracellular matrix as well. Importantly, the ability of this protein to inhibit vascular smooth muscle cell–mediated calcification in vitro suggests that osteopontin might be used in therapies aimed at preventing dystrophic calcification.

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