Identifying the processes and molecular mediators of atherosclerotic plaque formation are currently areas of intense investigation. While focal smooth muscle cell (SMC) accumulations have been considered a hallmark of atherosclerosis and restenosis, recent data suggest that the frequency of SMC replication in plaques is low. Thus, smooth muscle proliferation may not be a dominant event in lesion progression. Instead, other processes, such as cell migration, extracellular matrix formation, neovascularization, and calcification may be important pathophysiological events that are linked to the expression of genes unique to atherosclerotic plaque and not found in the normal vessel wall. Examples of molecules that are overexpressed in plaque compared with the normal vessel wall include platelet-derived growth factor-A, bone morphogenetic protein-2a, the major histocompatibility complex class II (1a) antigen, and the focus of the current study, osteopontin (OPN). Although the mechanisms by which these genes are regulated are unknown, it is plausible to consider that SMC diversity plays a role, since plaque SMCs show a spectrum of phenotypes ranging from well differentiated (ie, containing abundant smooth muscle contractile proteins) to relatively undifferentiated (ie, containing little contractile apparatus but increased protein synthetic machinery), with the latter being present in the greatest numbers and commonly implicated in lesion progression.

Using a differential cloning strategy, we have identified OPN, as well as several other genes (eg, collagen 1 [\(\alpha-1\)], elastin), that are overexpressed in the rat neointima in vivo and that distinguish rat vascular SMC phenotypes in vitro. OPN is an arginine-glycine-aspartate-containing acidic phosphoprotein normally restricted to bone matrix, kidney, and epithelial lining cells and has been implicated in bone morphogenesis, tumor metastasis, bacterial resistance, immune function, and renal physiology. In vitro, OPN serves as an adhesive substrate for both vascular smooth muscle and endothelial cells as well as a potent chemotactic factor for SMCs.

OPN protein is present focally in human atherosclerotic coronary and carotid artery specimens but absent in nondiseased coronary arteries. Although these studies suggest that OPN protein is overexpressed in the diseased intima compared with the normal vessel wall, they were not definitive because OPN mRNA transcripts were not measured. Since OPN circulates at low levels in healthy individuals, we believed it was critical to address this issue.

Abstract How an atherosclerotic plaque evolves from minimal diffuse intimal hyperplasia to a critical lesion is not well understood. Cellular proliferation is a relatively infrequent and modest event in both primary and restenotic coronary atherectomy specimens, leading us to believe that other processes, such as the formation of extracellular matrix, cell migration, neovascularization, and calcification might be more important for lesion formation. The investigation of proteins that are overexpressed in plaque compared with the normal vessel wall may provide clues that will help determine which of these processes are key to lesion pathogenesis. One such molecule, osteopontin (OPN), is an arginine-glycine-aspartate−containing acidic phosphoprotein recently shown to be a novel component of human atherosclerotic plaques and selectively expressed in the rat neointima following balloon angioplasty. Using in situ hybridization and immunohistochemical methods, we demonstrate that in addition to macrophages, smooth muscle and endothelial cells synthesize OPN mRNA and protein in human coronary atherosclerotic plaque specimens obtained by directional atherectomy. In contrast, OPN mRNA and protein were not detected in nondiseased vessel walls. Furthermore, extracellular OPN protein collocalized with sites of early calcification in the plaque that were identified with a sensitive modification of the von Kossa staining technique. These findings, combined with studies showing that OPN has adhesive, chemotactic, and calcium-binding properties, suggest that OPN may contribute to cellular accumulations and dystrophic calcification in atherosclerotic plaques.

Key Words • osteopontin • atherosclerosis • restenosis • coronary atherectomy • calcification

Osteopontin Is Synthesized by Macrophage, Smooth Muscle, and Endothelial Cells in Primary and Restenotic Human Coronary Atherosclerotic Plaques


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In the present study, we have correlated for the first time the appearance of OPN protein and mRNA in smooth muscle and endothelial cells as well as in macrophages found in advanced human atherosclerotic coronary lesions obtained by directional atherectomy. These studies clearly demonstrate that OPN is synthesized by cells within the plaque and suggest that these cell types contribute to the extracellular accumulation of plaque OPN. Combined with studies showing its adhesive and chemotactic properties, our data suggest that OPN may be a contributing element in the development of both primary and restenotic atherosclerotic lesions. In addition, extracellular OPN colocalized with sites of early calcium deposition in the plaque, and these studies provide further support for the role of this protein in the little-understood process of vessel wall calcification.

Methods

Coronary Artery Tissue Collection

Coronary artery and directional coronary atherectomy specimens were obtained from the Departments of Pathology at the University of Washington Medical Center and Sequoia Hospital after receiving ethical approval from the respective hospitals for the secondary use of this tissue. Nondiseased coronary artery segments were collected at the time of cardiac transplantation from three explant hearts of patients (aged 37, 50, and 57 years old) with nonischemic dilated cardiomyopathies. All three coronary artery segments had the typical noncritical diffuse intimal thickening common to adult coronary arteries and no evidence of advanced plaque complexity (eg, lipid accumulation, necrotic core, fibrous cap, or vasa vasorum). One critically diseased coronary artery lesion from the explant heart of a 57-year-old man with an ischemic cardiomyopathy was also included in the study.

Primary (9 male and 1 female; median age, 59 years; range, 45 to 78 years) and restenotic (7 male and 1 female; median age, 59 years; range, 45 to 75 years) coronary atherectomy specimens were obtained from nonconsecutive patients who underwent directional coronary atherectomy (Simpson Coronary Atherocath, Devices for Vascular Intervention, Inc) in the cardiac catheterization laboratories of the University of Washington Medical Center and Sequoia Hospital. Multiple fragments of atheromatous tissue weighing approximately 10 to 30 mg were obtained from each lesion. Only specimens with more than three tissue fragments or one to three large tissue fragments were included. Those specimens with fewer than four tissue fragments were included only if each tissue fragment was large and cellular. The number of tissue fragments in the primary versus restenotic specimens was similar (eg, median number of fragments, 5.1 versus 4.5 and mean number of fragments, 5.0 versus 4.5, respectively). From studies using image analysis (E.R.O.'B., unpublished data, 1993), we know that the total number of cells per slide on typical primary (n=18) and restenotic (n=19) atherectomy tissue slides is similar (eg, 5,520±1188 versus 4111±42 cells per slide, respectively; p=.48). All specimens were obtained from native coronary artery lesions. Diseased and nondiseased arterial tissue specimens were fixed in 10% neutral-buffered formalin, paraffin-embedded, processed according to conventional techniques, and cut into 5-μm thick serial sections that were placed on Superfrost Plus glass slides (Fisher Scientific). A hematoxylin and eosin- as well as a Verhoeff-van Gieson-stained slide was made for each specimen.

In Situ Hybridization

OP-10 is a 1493-bp human OPN cDNA isolated from primary cultures of human bone cells and subcloned into pBluescript SK plasmid (a generous gift from Dr L. Fisher, National Institutes of Health). 32P-UTP-labeled antisense and sense riboprobes were generated with T7 or T3 polymerase after linearizing the plasmid with Xba I and Xho I, respectively. Labeled riboprobe transcripts were separated from unincorporated precursors by using a Nick column (Pharmacia). After precipitating the labeled probe in ethanol, the probe was resuspended in hybridization buffer to yield aliquots containing 300 000 cpm/μL. Slides from each tissue specimen were hybridized with antisense and sense riboprobes at 55°C overnight. The slides were then washed briefly in 2× SSC followed by 0.1× SSC for 2 hours at 55°C, after which they were dipped in Kodak NTB2 emulsion and stored in the dark at 4°C in desiccated boxes. All slides were developed at 15°C 7 days after hybridization. Because OPN is expressed constitutively by tubular epithelial cells, normal human kidney was used as a positive control tissue. The amount of in situ signal detected on the atherectomy slides was assessed semiquantitatively by using the following grading system: grade 0, ≤5 grains overlying the nuclei of <10 cells per tissue fragment (background); grade 1+, ≤5 grains overlying the nuclei of ≤10 cells per fragment; grade 2+, 1 to 2 tissue fragments per slide, each with ≥10 cells that have ≥5 grains overlying each nucleus; and grade 3+, ≥3 tissue fragments per slide, each with ≥10 cells that have ≥5 grains overlying each nucleus (Table 1). The in situ hybridization results of one diseased and three nondiseased coronary arteries were summarized in a descriptive manner.

Immunocytochemistry

Adjacent slides were designated for immunocytochemical labeling for the OPN protein as well as cell identity markers. Briefly, this involved deparaffinizing sections, blocking endogenous peroxidase activity with H2O2, and applying the specific primary antibody on a slide for 60 minutes at room temperature. LF-7, a rabbit antiserum directed against human bone OPN (kindly donated by Dr L. Fisher, National Institutes of Health), was used to immunolabel human OPN protein in the vessel wall (titer, 1:1000). A biotinylated anti-rabbit secondary antibody was then applied for 30 minutes, followed by an avidin-biotin-peroxidase conjugate (ABC Elite, Vector Laboratories) for 30 minutes at room temperature. Standard peroxidase enzyme substrate, 3,3’-diaminobenzidine (DAB) with or without nickel chloride was then added to yield either a brown or black reaction product, respectively. For each LF-7 immunocytochemistry run, the human kidney used as a positive control consistently displayed dark black immunolabeling of tubular epithelial cells a reaction product; we have defined this as our standard of positivity. The following antibodies were used as cell identity markers: anti-smooth muscle α-actin (1:100 dilution; Boehringer Mannheim Corp) to identify SMCs, anti-CD-68 (1:50 dilution; DAKO-CD68, KPI, DAKOPATTS; predigested with 1 x trypsin for 20 minutes) to identify macrophages, and anti-HPCA-1 (CD-34) antibody (1:34 dilution; Becton Dickinson Immunocytochemistry Systems; predigested with 1 x trypsin for 20 minutes) to identify endothelial cells. These cell identity markers were then detected with a biotinylated anti-mouse secondary immunoglobulin G antibody, followed by an avidin-biotin-peroxidase conjugate (as described above) and DAB to yield a brown reaction product. Hematoxylin or methyl green was used as nuclear counterstain.

The degree of OPN cellular immunolabeling was semiquantified by using the following grading system: grade 0, no immunolabeling detectable; grade 1+, positive immunolabeling for <10 cells in one tissue fragment; grade 2+, 1 to 2 tissue fragments each with ≥10 cells labeled positively; grade 3+, ≥3 tissue fragments, each with ≥10 cells labeled positively (Table 2).

Colocalization of OPN Protein and Plaque Calcium

To demonstrate the spatial association between OPN protein and areas of plaque calcification, adjacent slides were...
TABLE 1. Expression of Osteopontin mRNA and Protein In Coronary Atherectomy Tissue

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Hybridization Grade</th>
<th>mRNA Localization</th>
<th>Immunocytochemistry Grade</th>
<th>Protein Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>1+</td>
<td>SMC</td>
<td>2+</td>
<td>MΦ AND SMC</td>
</tr>
<tr>
<td>Primary</td>
<td>2+</td>
<td>SMC</td>
<td>3+</td>
<td>SMC</td>
</tr>
<tr>
<td>Primary</td>
<td>1+</td>
<td>SMC</td>
<td>2+</td>
<td>SMC</td>
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<tr>
<td>Primary</td>
<td>3+</td>
<td>MΦ</td>
<td>2+</td>
<td>MΦ</td>
</tr>
<tr>
<td>Primary</td>
<td>1+</td>
<td>SMC AND MΦ</td>
<td>2+</td>
<td>SMC</td>
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<tr>
<td>Primary</td>
<td>3+</td>
<td>MΦ</td>
<td>3+</td>
<td>MΦ</td>
</tr>
<tr>
<td>Primary</td>
<td>3+</td>
<td>SMC, MΦ, AND EC</td>
<td>2+</td>
<td>SMC</td>
</tr>
<tr>
<td>Primary</td>
<td>2+</td>
<td>SMC</td>
<td>2+</td>
<td>SMC</td>
</tr>
<tr>
<td>Primary</td>
<td>2+</td>
<td>SMC, MΦ, AND EC</td>
<td>2+</td>
<td>MΦ</td>
</tr>
<tr>
<td>Primary</td>
<td>3+</td>
<td>SMC</td>
<td>3+</td>
<td>SMC</td>
</tr>
<tr>
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<td>MΦ</td>
<td>2+</td>
<td>MΦ</td>
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<td>...</td>
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<td>SMC AND MΦ</td>
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<td>SMC</td>
<td>1+</td>
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<tr>
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<td>1+</td>
<td>SMC AND MΦ</td>
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<td>MΦ AND EC</td>
<td>2+</td>
<td>M AND EC</td>
</tr>
<tr>
<td>Restenotic</td>
<td>3+</td>
<td>SMC=MO</td>
<td>3+</td>
<td>SMC=MO</td>
</tr>
</tbody>
</table>

SMC indicates smooth muscle cell; MΦ, macrophage; and EC, endothelial cell.

*Increasing expression of osteopontin mRNA: 0 to 3+.
†Increasing expression of osteopontin protein: 0 to 3+.

used for LF-7 immunolabeling and a Von Kossa stain. The Von Kossa stain yields a black color reaction due to the precipitation of silver phosphates and carbonates, which are associated with calcium in mineralized deposits. Soluble phosphates and carbonates are washed out during tissue processing and the Von Kossa staining procedure, leaving only calcium phosphates and calcium carbonates to react with the silver cation. Unfortunately, the Von Kossa stain is best performed on tissue that is fixed in alcohol rather than formalin. Therefore, 19 coronary atherectomies (primary: 8 male and 2 female; median age, 65 years; range, 45 to 75 years; restenotic: 8 male and 1 female; median age 63 years; range 42 to 78 years) that had been fixed in methyl Carnoy’s fixative (glacial acetic acid/methanol/chloroform, 10:60:30, vol/vol/vol) were used for this purpose. For this analysis the average number of tissue fragments per slide was 8.9. The OPN immunolabeling protocol was identical to that used for the formalin specimens as described above. For the Von Kossa staining, atherectomy slides were deparaffinized and rehydrated before being immersed in a light-protected, silver nitrate solution (5.0 g in 100 mL distilled water) for 30 minutes. The slides were then immersed in D-19 Kodak developer for 15 minutes while exposed to the light from a 100-W bulb, followed by immersion in a solution of 5% sodium thiosulfate in distilled H₂O for 2 minutes. Finally, the slides were counterstained with nuclear fast red.

Adjacent slides with Von Kossa staining and OPN immunolabeling were then reviewed by light microscopy at 100× to determine the spatial relation between OPN and plaque calcification (Table 2). A map of all tissue fragments on each slide was drawn, and the presence or absence of OPN immunolabeling and/or the black color reaction of the Von Kossa stain was recorded. The gross histology of the tissue fragments was also noted (eg, acellular extracellular matrix versus cellular regions). Because directional atherectomy results in the recovery of multiple tissue fragments of various sizes and

TABLE 2. Immunodetectable Osteopontin Protein and Calcium In Coronary Atherectomy Specimens

<table>
<thead>
<tr>
<th></th>
<th>Primary</th>
<th>Restenotic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of specimens</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>No. of fragments</td>
<td>88</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Osteopontin-positive fragment</td>
<td>74 (84%)</td>
<td>52 (63%)</td>
<td>NS</td>
</tr>
<tr>
<td>Osteopontin-negative fragment</td>
<td>14 (16%)</td>
<td>30 (37%)</td>
<td>.04</td>
</tr>
<tr>
<td>Calcified zones</td>
<td>36 (41%)</td>
<td>14 (17%)</td>
<td>.01</td>
</tr>
<tr>
<td>Osteopontin-positive zone</td>
<td>35 (97%)</td>
<td>12 (86%)</td>
<td></td>
</tr>
<tr>
<td>Osteopontin-negative zone</td>
<td>1 (3%)</td>
<td>2 (14%)</td>
<td>NS</td>
</tr>
</tbody>
</table>
composition, we selected slides with multiple (mean, 8.9) tissue fragments per slide. It was not uncommon to find more than one discrete focus of calcification in some of the larger fragments. Therefore, discrete areas of calcification were counted as separate calcified zones within the same tissue fragment.

### Statistical Analysis

The data in Table 2 compare primary and restenotic tissue, particularly discrete tissue areas that are calcified or have anti-OPN immunolabeling. To ensure that individual tissue areas from one patient could be considered as independent entities, the data were evaluated by using the method of generalized estimating equations, which accounts for potential dependence between repeated observations on the same object. Statistical significance was defined as \( P<.05 \).

### Results

#### In Situ Hybridization

OPN mRNA was not detected by in situ hybridization in three coronary artery cross sections showing diffuse intimal hyperplasia without evidence of atherosclerosis (Fig 1). Conversely, one critically stenosed coronary artery had several areas with significant OPN antisense riboprobe labeling in the intima and a lesser amount in the media. Table 1 outlines the distribution of OPN mRNA (and protein) in both primary and restenotic coronary atherectomy specimens. Except for two restenotic specimens, all coronary atherectomy specimens had detectable OPN antisense riboprobe labeling (Table 1). Adjacent slides showing cell-identity markers were reviewed to determine which cells were making OPN mRNA. Expression of OPN mRNA was observed in the following histological patterns: (1) collections of macrophages bordering on dense connective tissue, similar in appearance to the typical description of the fibrous cap (Fig 2); (2) uniform areas of stellate-shaped SMCs devoid of other cell types (Fig 3); (3) areas of SMCs with rounded cell contours intermixed with macrophages; and (4) three specimens with microvessels present in the plaque that had evidence of OPN expression overlying the vessels, thereby suggesting that endothelial cells may also make OPN. No specific signal was observed when the OPN sense riboprobe was applied to an adjacent slide for each specimen.

#### Immunocytochemistry

Specific cellular labeling for OPN protein was absent in the three coronary arteries with diffuse intimal thickening (Fig 1). In contrast, one critically diseased coronary artery did show widespread immunolabeling of OPN protein in the intima. All coronary atherectomy specimens had detectable levels of OPN immunolabeling (Table 1). OPN protein was found in macrophages and SMCs with patterns that correlated with the expression of the mRNA (Figs 2 and 3). Likewise, OPN protein was clearly found in endothelial cells of several but not all plaque microvessels that were present in one specimen (Fig 3). Relatively acellular areas of dense connective tissue reminiscent of the fibrous cap (Fig 2) and areas of calcification (Fig 4) were also strongly positive for immunodetection of OPN protein. Interestingly, two specimens indicated in Table 1 displayed immunolabeling for OPN protein in both SMCs and macrophages, yet had no evidence of the mRNA by in situ hybridization. Conversely, the protein was not always as abundant as the mRNA. For example, in some areas of tissue consisting of predominantly stellate-shaped SMCs, mRNA was readily detected by in situ hybridization, but only modest amounts of extracellular protein were detectable by immunolabeling (Fig 3). This may reflect a difference in half-lives between the OPN mRNA and protein in these cells. Furthermore, proteolytic events are thought to be highly active in atherosclerotic plaques and may have contributed to these findings.

#### Colocalization of Plaque Calcium and OPN Protein

Ten primary and nine restenotic coronary atherectomy specimens (comprising 88 and 82 tissue fragments, respectively) were analyzed for OPN immunolabeling and the presence of plaque calcification (Table 2). Immunodetectable OPN protein was more commonly found in primary than in restenotic tissue fragments (74 [84%] of 88 primary versus 52 [63%] of 82 restenotic tissue fragments; \( P=.04 \)). Plaque calcification was more commonly detected in primary than in restenotic tissue fragments (36 [41%] of 88 primary versus 14 [17%] of 82 restenotic tissue fragments; \( P=.01 \)). In general, large extracellular deposits of calcium in hypocellular dense connective tissue were more common than speckled calcification in areas of hypercellularity. Immunodetectable OPN protein colocalized with tissue calcification in the majority of both primary and restenotic tissue fragments (35 [97%] of 36 primary and 12 [86%] of 14 restenotic calcified tissue areas; \( P<.05 \)).

#### Discussion

In the present study we found OPN mRNA and protein to be abundant in pathological atherectomy specimens derived from both primary and restenotic
Fig 3. Restenotic coronary atherectomy specimen from the right coronary artery obtained 7 months after the initial atherectomy procedure. A, The majority of the cells in the center of this tissue fragment are stellate-shaped; on a serial slide they immunolabeled with an anti-smooth muscle cell α-actin antibody (not shown) (hematoxylin-eosin, original magnification x100). B, Darkfield microscopy of tissue fragment from panel A hybridized with antisense riboprobe to osteopontin (original magnification x100). Note the focal intense signal at the center of the tissue, in the region of the smooth muscle cells. C, Immunocytochemistry for osteopontin reveals modest amounts of the immunopositive brown color reaction in the smooth muscle cells of this particular specimen (original magnification x1000). D, Immunolabeling for osteopontin protein in endothelial cells of plaque microvessels (original magnification x1000). E, Adjacent tissue section immunolabeled with anti-CD34 antibody (black color reaction, original magnification x1000). Therefore, cells shown in panel D with immunodetectable osteopontin protein are endothelial cells of intraplaque microvessels.

Fig 4. Colocalization of osteopontin protein and plaque calcification in atherectomy tissue from a primary lesion of the left anterior descending coronary artery (original magnification x200). A, Modified Von Kossa stain with nuclear fast-red counterstain. Black areas represent calcium deposits in the plaque. B, Immunodetection of osteopontin in the same tissue. Note speckled appearance of the osteopontin protein distribution, which resembles that of the plaque calcification.
coronary artery lesions but undetectable in the media or intima of nondiseased coronary arteries. While macrophages appeared to be the major synthesizer of OPN, we also found that SMCs and endothelial cells contribute to plaque OPN production. Of particular interest, extracellular OPN protein was often found in areas of dense connective tissue, especially when calcification was present.

Prior to the completion of this study, Hirota and colleagues reported macrophage expression of OPN mRNA in human atherosclerotic aortas but not nondiseased vessel walls. Our findings partially agree with these results. However, unlike those investigators, who localized OPN mRNA exclusively in macrophages, we report for the first time the abundant OPN expression in plaque smooth muscle and endothelial cells. Several factors may account for this discrepancy. First, the riboprobe used by Hirota et al consisted of a 336-bp fragment of the published human OPN cDNA sequence, whereas we used the complete 1.4-kb sequence of human OPN. Second, Hirota et al used the digoxigenin colorimetric method to label their riboprobe, which is less sensitive than radioactively labeling the cDNA, and may be another possible reason for their limited results. Furthermore, because these investigators did not use positive control tissue to assess the hybridization signal of their labeled probe, it is impossible to assess the sensitivity of their labeled riboprobe. Third, we examined 18 advanced atherosclerotic coronary artery specimens from symptomatic patients, whereas Hirota and colleagues studied six human aortas with modest atherosclerotic disease. In the absence of complex plaque (eg, necrotic core, lipid infiltration, neovascularization), these investigators may have lacked the pathological substrate for OPN expression, which would account for their limited detection of OPN in plaque macrophages but not SMCs or endothelial cells. Finally, Hirota and colleagues did not perform immunocytochemistry for OPN protein detection. The results from our OPN immunodetection were consistent with in situ hybridization data, showing the widespread distribution of OPN in macrophages and SMCs as well as the endothelial cells of a few plaque microvessels.

Why might OPN expression be unique to the plaque and not be found in the normal vessel wall? First, it should be noted that OPN was first identified as a vessel wall product when we found overexpression of its mRNA in phenotypically distinct SMCs derived from pup rat aortas, which resemble neointimal cells in growth properties, morphology, and gene expression patterns. This finding is supported by the work of Shanahan and colleagues, who found OPN to be preferentially expressed in dedifferentiated, late-passage SMCs compared with freshly dispersed, differentiated, rat aortic SMCs. Furthermore, Gadeau et al found OPN to be expressed during cell cycle mid-G1 phase in proliferating vascular SMCs in vitro. Therefore, OPN expression in plaque may be due to the presence of uniquely differentiated SMCs. A second possible explanation for the overexpression of OPN in plaque compared with the normal vessel wall might be the influence of local cytokines or growth factors in the plaque but not the normal vessel wall. In vitro studies indicate that OPN expression is responsive to a wide range of growth factors that may be present at sites of arterial injury. In particular, OPN mRNA and protein levels are increased in rat vascular SMCs treated with angiotensin II, basic fibroblast growth factor, transforming growth factor-β, and platelet-derived growth factor. In either case, it is important to emphasize that OPN is not found in the normal media, thereby implying that the SMCs of primary and restenotic coronary atherectomy tissue somehow differentially regulate this gene. Furthermore, it is noteworthy that calcification and OPN protein were more prevalent in primary than in restenotic coronary atherectomy specimens. The significance of these findings is uncertain, but they may reflect differences in the biology of primary and restenotic lesions or they may be simply an artifact of how these specimens are collected (eg, primary tissue may contain more superficial, fibrocalcific mass, while restenotic samples may come from hypercellular areas deeper in the artery wall).

This study clearly demonstrates for the first time that endothelial cells can, under certain circumstances, synthesize OPN. In support of this, other investigators have anecdotally noted OPN immunostaining or in situ signals in undefined structures most likely to be capillary vessels of the endometrium and brain. Furthermore, in preliminary studies, we found OPN expression to be minimal in cultured bovine aortic endothelial cells, yet dramatically upregulated in response to scratch injury. OPN has been correlated with bone formation and repair processes and expression of OPN in an animal model of renal angiosclerosis suggests that OPN may play a role in plaque calcification. In this study we used a sensitive modification of the Von Kossa stain to detect early sites of calcium deposition. OPN was colocalized at these sites, suggesting that it may play an early role in the calcification process. Little is known about the biology of arterial wall calcification; however, Bostrom et al re-
port the expression of bone morphogenetic protein–2a, a potent factor for osteoblastic differentiation, in human atherosclerosis and suggest that plaque calcification is an active process. Our findings that three different cell types in the wall actively synthesize OPN, another protein implicated in bone formation, support this contention.

Plaque calcification may have several important clinical implications. Current images with ultrafast computed tomography suggest that coronary artery calcification is associated with the presence of clinically significant lesions. Furthermore, lesion calcification has an important influence on the deployment and success of the growing number of catheter-based mechanical coronary interventional procedures. For example, plaque calcification can limit the inflation of coronary angioplasty balloons or the ability of a directional atherectomy device to cut tissue. Alternatively, dissection planes often originate at the junction of calcified and noncalcified tissue and have been associated with an increased risk of subsequent adverse events. The data from this study suggest that calcification is frequently found in coronary atherectomy specimens, with primary specimens being more calcified than restenotic samples.

How might these data mesh with our current understanding of primary coronary artery disease and restenosis? Low levels of SMC proliferation have been detected in diseased coronary arteries by using immunocytochemistry for the proliferating cell nuclear antigen. Conversely, the problem of restenosis that commonly occurs after coronary interventional procedures has been thought to be primarily due to SMC proliferation. More recently, however, we have documented only modest and infrequent levels of proliferation in primary and restenotic coronary atherectomy specimens by using immunocytochemistry for the proliferating cell nuclear antigen. Hence, it appears that SMC proliferation may be less critical to lesion progression or restenosis than other processes, such as connective tissue synthesis, cell migration, neovascularization, inflammation, and calcification. All of these events may depend on the presence of molecules that are overexpressed in the intima compared with the normal vessel wall, and OPN may be one such molecule. We hope that further studies of the functional role of OPN in the vessel wall will lead to the identification of mechanisms by which this molecule is associated not only with atherogenesis but also vessel wall calcification.

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