J Korean Med Sci 2000; 15: 485-93
ISSN 1011-8934

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Expression of Osteopontin in Calcified Coronary Atherosclerotic Plaques

Advanced atherosclerosis is often associated with dystrophic calcification and remodeling of extracellular matrix of vascular wall. Recently many studies have documented a general relationship between calcification and severity of coronary disease, and discussed the feasibility of electron beam computed tomography for detecting and quantifying the coronary artery calcification in the patients. The present study investigated the expression and the localization of osteopontin, one of noncollagenous bone matrix protein, within the calcified coronary arteries. Autopsy-derived coronary artery specimens were scanned and reconstructed to visualize the pattern of coronary calcification using a novel microscopic computed tomography technique. The localization of the osteopontin were evaluated by immunohistochemial stain with LF7. The present study showed that the pattern of coronary calcification is variable and the expression of osteopontin is localized mainly to calcified lesion. The smooth muscle cells in addition to macrophage expressed osteopontin protein in human coronary atherosclerotic plaques. Soluble osteopontin released near to the sites of vascular calcification may represent an adaptive mechanism aimed at regulating the process of vascular calcification.

Key Words: *Coronary Artery Calcification; Osteopontin*

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Received: 8 May 2000
Accepted: 20 July 2000

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INTRODUCTION

Until recently, atherosclerosis had been regarded as a degenerative process, but extensive research has demonstrated its active regulation of cellular and extracellular elements in, for example, the vascular calcification. O'Brien et al. provided evidence that valvular as well as vascular calcification are an active, regulated processes with similarities to bone formation and remodeling (1, 2).

New imaging techniques made it clear that coronary calcification is neither rare nor a final stage, occurring in 90% of all patients with coronary disease (3), and that the vast majority of significant coronary stenosis are calcified (4). Recent histopathologic analysis of human carotid arterial atherosclerosis has shown that intraplaque hemorrhage-thrombosis might contribute to arterial calcification as a source of bone matrix proteins and that apolipoproteins, fibrin and matrix metalloproteinases might interact in the formation and progression of dystrophic calcification (5). Coronary calcification is also associated with atherosclerotic lesions increasing cardiovascular risk (6-10).

In coronary artery specimens Fitzpatrick et al. (11) identified mRNA of matrix proteins associated with mineralization. Specifically they identified a cell attachment protein (osteopontin) and a γ-carboxylated protein that regulates mineralization (osteocalcin). Osteopontin (OPN) is an acidic, phosphorylated glycoprotein, which is regulated by local cytokines and is known as inhibitor of bone formation and calcification. OPN was named for its function as a bridge between cells and mineral. Forming a proteinaceous coating over the solid crystal surface, OPN mediates attachment of both osteoblasts and osteoclasts to bone mineral through interaction of its highly conserved GRGDS sequence with intergrins (12, 13). OPN is abundant at sites of calcification in human atherosclerotic plaques and in calcified aortic valves, and OPN mRNA appears to be expressed predominantly in lesion monocyte/macrophages but also in vascular smooth muscle cells (1, 2, 14-18).

Although a prominent component of the extracellular matrix of bone, the function of OPN in hard tissue formation, mineralization, and turnover is not yet clear. Several studies have suggested that osteopontin may be an important regulator of vascular calcification (13, 19-23) and also a chemoattractant factor released by acti-

vated vascular smooth muscle cells to guide the migration of fibroblasts (24). Recently, Wada et al. (23) demonstrated that soluble OPN released near to the sites of vascular calcification may represent an adaptive mechanism aimed at preventing vascular calcification. Paradoxically, bone matrix proteins that inhibit apatite formation are found at increased levels in calcified human atherosclerotic plaque and in culture (17, 23, 25).

Prior pathologic (26, 27), angiographic (28, 29) and intravascular ultrasound studies (30) have not specifically examined the pattern of vascular calcification and the characterization of calcific tissues. A growing number of investigations (3, 31, 32) has demonstrated the feasibility of using electron beam computed tomography (EBCT) scanning for detecting and quantifying the coronary artery calcification in vivo. In the present study, Microscopic computerized tomography (Micro-CT) allowed the assessment of the three-dimensional pattern of vascular structure in vitro and provided a useful means for the study of the spatial distribution of calcification within the vessel wall (33-37).

The purpose of the present study was to evaluate the characterization of calcific tissue within the vessel wall and the expression of OPN in autopsy-derived coronary artery specimens of non-cardiac deaths.

MATERIALS AND METHODS

Study population

Human coronary samples were collected from autopsies within 24 hr postmortem (two men, aged 71 and 69 years; two women, aged 58 and 65 years). Of the four individuals, 2 died from metastatic carcinoma, 1 from renal failure, and 1 from sepsis. Two patients with diabetes, one patient with hypercholesterolemia, and three patients with hypertension were documented in this series of patients. Two patients were smokers at the time of death.

Tissue preparation and polymer injection

Following removal of the heart, glass cannulae were tied at the left coronary orifices and injected with 500 mL of heparinized saline (0.9% sodium chloride with 5,000 units of heparin) at a pressure of 70 mmHg to clear the coronary network of remaining blood. A specially prepared, low viscosity, radio-opaque liquid polymer compound (MV-122, Canton Biomedical Products, Boulder, CO, U.S.A.) was then injected through the cannulae. The heart was then immersed in 10% buffered formalin and placed under refrigeration at 4℃ overnight to allow polymerization of the plastic compound. On the following day, the coronary arterial segments (about 2 cm in the length) were removed from the heart by careful dissection, and the luminal polymer was removed to differentiate vascular calcification from radio-opaque polymer. The coronary segments were placed in 70% ethanol and dehydrated through increasing alcohol concentrations. At successive 24-hr intervals, glycerin concentration was raised from 30 to 50, 75 and finally 100% glycerin in order to completely dehydrate the coronary segments. The specimens were then rinsed in acetone, and left in open air for 24 hr. Finally, the coronary segments were embedded in wax for scanning and three-dimensional (3D) reconstruction. After micro-CT reconstruction and analysis, coronary specimens were immersed for 4 hr in water at 40℃ to gently melt the wax embedding, were removed from the plastic mold, and were cut at 3 mm intervals along the entire length of all arteries for histopathologic studies.

Right coronary arteries were not injected with radio-opaque polymer and the coronary arterial segments (about 2 cm in the length) were removed from the heart for immunochemistry.

Microscopic three-dimensional CT reconstruction and ultrastructural imaging

Micro-CT was utilized for ultrastructural visualization of the plaque calcification. Specimens were scanned by a micro-CT system consisting of a spectroscopy X-ray tube, a fluorescent crystal plate, a microscopic objective and a charge coupled device camera (CCD) (33-37). The charge in each pixel was digitized and stored in a computer as an array of 500-1000 projections of the specimen in 360-degree rotations.

Three-dimensional (3D) images were reconstructed using a modified Feldhaup cone beam filtered back projection algorithm and the resulting 3D images were displayed using the Mayo Analyze software (Version 7.5, Biomedical Imaging Resource, Mayo Foundation). Volume rendering provided a variety of display representations of 3D image data sets. Volume rendered transmission displays, cross-sectional, maximum intensity projection (gray scale/inverted gray scale) and curved sectional images were displayed at various angles and threshold values of voxels. Average voxel size was 21-28 mm, and images of up to 800 slices were rendered for each arterial specimen (each with a matrix of 10-20 mm cubic voxels $\times$16 bits of gray scale).

The cross-sectional images were analyzed at every 3 mm of each specimen first at the pixel size of 28 μm and then at higher resolution using 21 μm voxel size. The cross-sectional and curved sectional micro-CT images

were captured and digitized in the computer and an intensity histogram was made to display the distribution of gray levels (from black to white) within the image. Calcific areas were differentiated against the black background by setting the upper and the lower threshold values for an intensity range of interest (IROI) that yielded the best identification of calcific regions as judged by the operator.

Immunohistochemistry

Immunohistochemistry was performed with LF7, which is a rabbit polyclonal anti-osteopontin antiserum raised against human bone-derived osteopontin (NIH, Bethesda, MD, U.S.A.) and was previously used for immunohistochemistry on human arterial tissue, was used at a titer of 1:1,000 to localize the osteopontin protein. Peroxidase-conjugated secondary antibodies were used with the primary antibody.

Paraffin sections (5 μm) were made and transferred to glass slides. The paraffin sections were deparaffinized and rehydrated through xylene for three times and for 3 min each, and through serial concentrations of 100, 95, 80, and 75% ethanol for 3 min each. The sections were boiled with citric acid for 5 min to suppress nonspecific binding of the antibodies and to increase the exposure of antigens, and were cooled at room temperature for 20 min. The sections were then treated with 0.3% H_2O_2 for 5 min to suppress the endogenous peroxidase activity. After treatment with TBS (pH=7.2-7.4), the sections were incubated in moist chamber for 1 hr with primary antibodies. The sections were then processed by the streptavidin-biotin-peroxidase complex method by use of the LSAB plus kit (DAKO Inc., Carpenteria, CA, U.S.A.) and stable DAB solution (Research Genetics Inc., Huntsville, AL, U.S.A.) to produce a brown color at the site of reactivity. The sections were then counterstained with methylene blue.

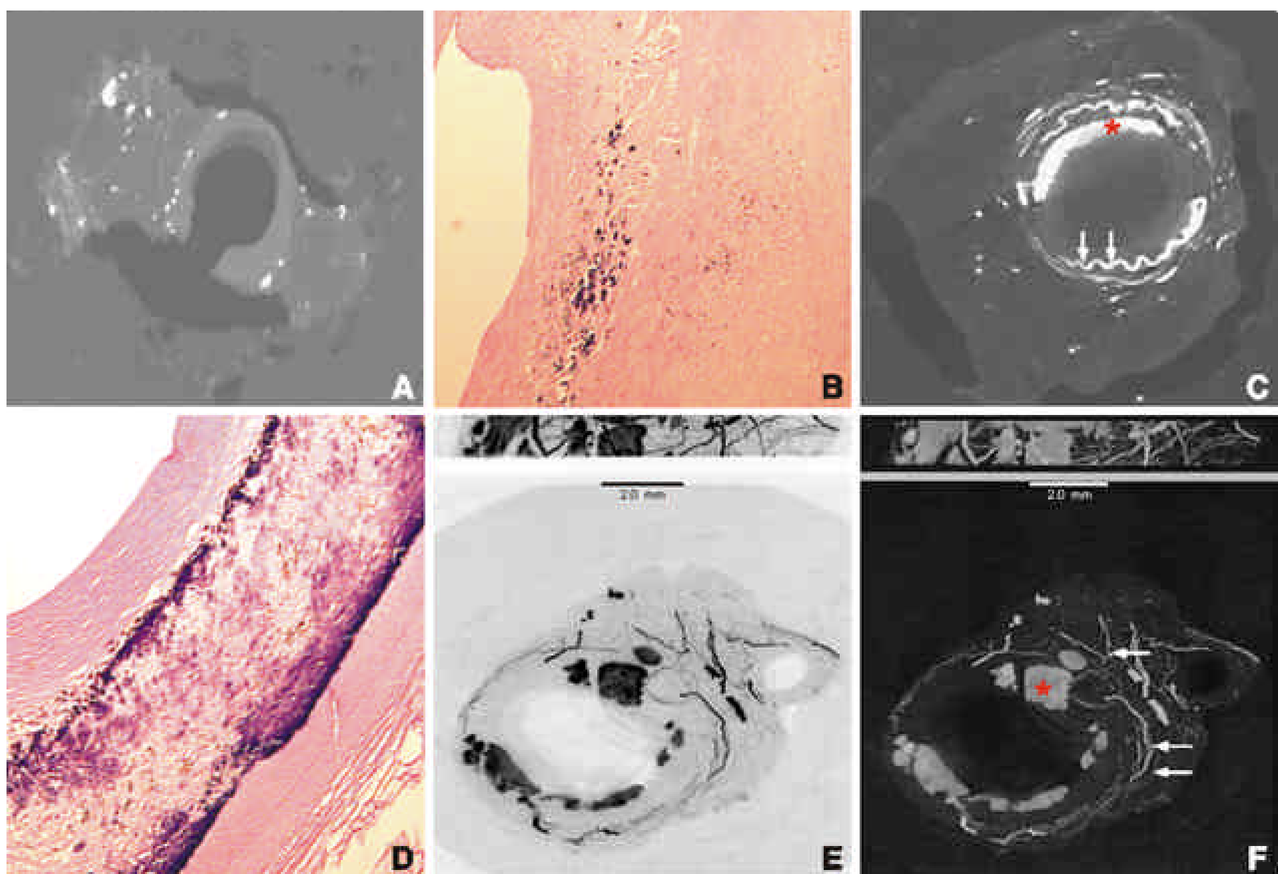

Fig. 1. High-resolution micro-CT and histologic (H&E, ×10) cross-sectional images from discrete (**A** and **B**, Case 1) to heavy calcification (asterisk) including the intima, media and elastic lamina (**C** and **D**, Case 2) of human coronary arteries (voxel size 21 mm). Calcified elastic lamina (arrow in **C**) is located between calcified plaque and medial calcification (**D**). Maximum intensity projection, inverted gray scale (**E**, Case 3) and gray scale (**F**, Case 3) demonstrat that preserved vessel lumen is accompanied with increasing thickness of the calcific wall and neovascularized vasa (long arrow) in the outer region of the vessel wall in human calcific coronary artery (bar=2.0 mm).

RESULTS

Cross-sectional images demonstrated that the patterns of coronary calcification associated with atherosclerosis were variable, being discrete or focal (Fig. 1A, 1B) and linear or circumferential. The circumferential or linear structures (Fig. 1C, 1D) were identified as elastic lamellae and medial calcification. The medial calcification was also observed in H&E stained sections of the coronary arteries (Fig. 1D), which revealed calcification underlying the intimal hyperplasia. These findings demonstrated that in the presenting cases of muscular arteries, deep calcification might be associated with elastic lamella. Maximum intensity projection and inverted gray scale/gray scale (Fig. 1E, 1F) demonstrated that the preserved vessel lumen accompanied calcific walls of increasing thickness and neovascularized vasa vasorum within plaque itself in human calcific coronary artery.

Comparing with the corresponding sections in Fig. 1C, extensive calcium is deposited relatively uniformly along the non-critical stenosis of plaque, as shown by maximum intensity projection and by curved sectional images (Fig. 2A, 2B). These findings may be due to vascular remodeling, where arteries increase in size to compensate for the atherosclerotic plaque growth and thus to preserve lumen size. In contrast, a large plaque with critical stenosis shows the neovascularization within vessel wall and plaque itself by micro-CT (Fig. 2C, 2D). Longitudinal sections of coronary arteries contained radio-opaque regions of calcification that were intervening between the non-calcified plaque as a focal pattern or linear structure along the intima and media (Fig. 2C, 2D).

Much of OPN protein was expressed in the cytoplasm of macrophages and smooth muscle cells in plaques and in extracellular areas along the calcification front (Fig. 3A, 3B). The calcification front or the fibrotic portion of the lesions in a granular pattern was composed of macrophages/foam cells, smooth muscle cells and cholesterol crystals (Fig. 3B). However, despite the higher expression of OPN in the front of calcification, especially in plaque itself and mediointimal junction, a subset of vascular smooth muscle cells in the outer media demonstrated the lower expression of OPN (Fig. 3A, 3B). Non-calcified atherosclerotic lesions revealed little OPN immunoreactivity in intimal smooth muscle cells and infiltrated inflammatory cells (Fig. 3C). Comparing with non-calcified lesion, calcified atherosclerotic plaque demonstrated marked increases in OPN immunoreactivity. A higher-magnification view of the front area of calcification in this lesion showed cytoplasmic localization of OPN in macrophages/foam cells and smooth muscle cells (Fig. 3D-F).

DISCUSSION

The results from the present study suggest the following conclusions regarding the coronary artery calcification and the expression of bone matrix protein OPN. Micro-CT is a novel and powerful in vitro technique that permits the assessment of the three-dimensional pattern of vascular structure and provides a useful mean for the study of the spatial distribution of calcification within the atherosclerotic plaque. OPN is abundant at sites of calcification in human atherosclerotic plaques and it appears

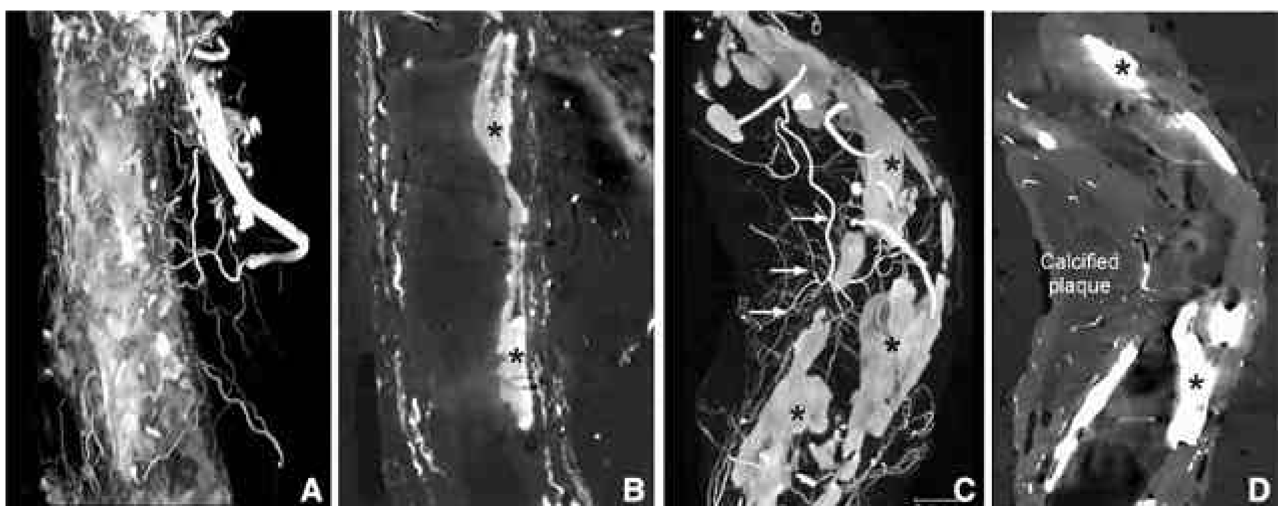

Fig. 2. Photomicrographs of calcified human coronary arteries. **A** and **B** (Case 2). Extensive calcium (asterisk) is deposited relatively uniformly along the non-critical plaque, as shown by maximum intensity projection (**A**, voxel size 21 mm) and by curved sectional image (**B**, L: lumen, P: plaque). **C** and **D** (Case 3). In contrast, a large plaque with near-total luminal occlusion shows the neovascularization within vessel wall (arrow) and plaque itself and a rim of microfocal mineralization by micro-CT (bar=2.0 mm).

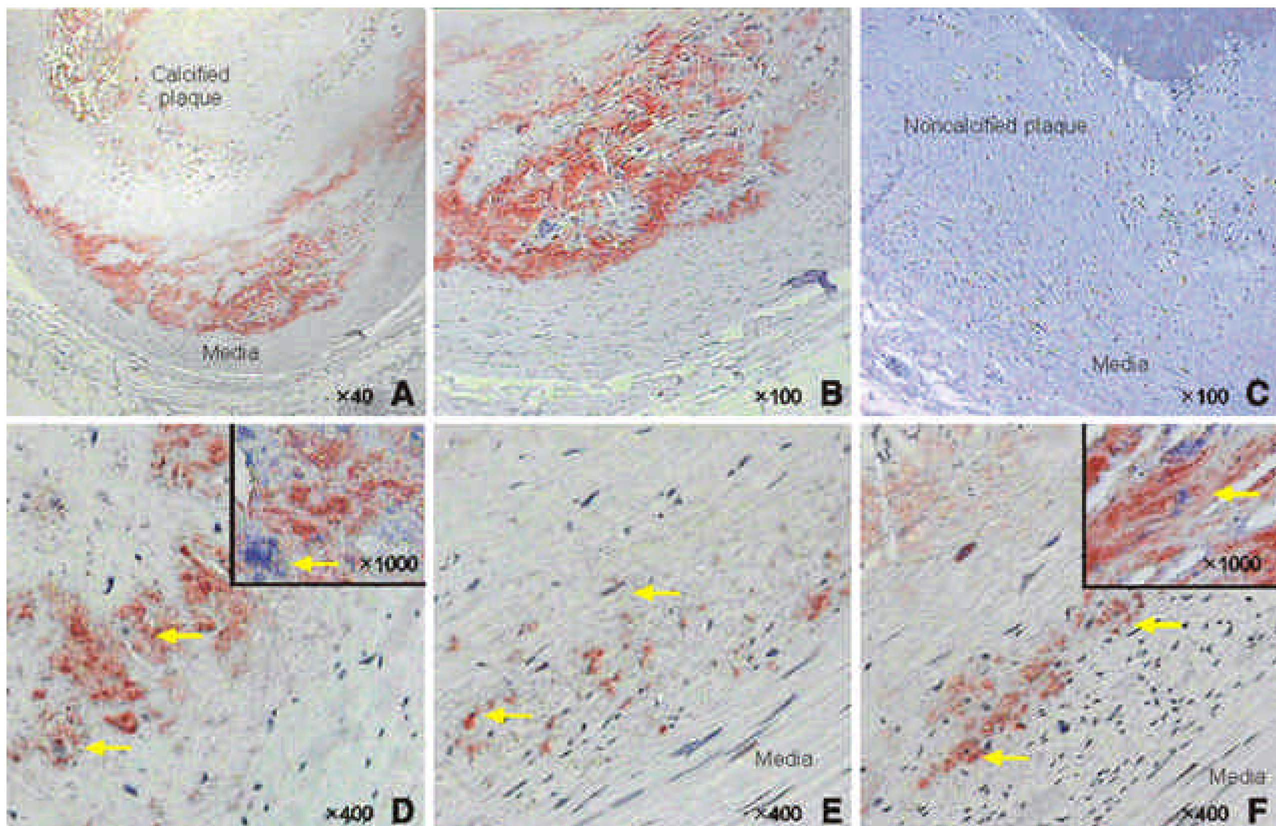

Fig. 3. Advanced atherosclerotic coronary section (A, Case 3, ×40) shows that severe calcified and total occluded lesion is associated with macrophages and cholesterol crystals. Osteopontin is expressed along the calcification front (B, Case 3, ×100). Comparing with calcified lesion, non-calcified atherosclerotic lesions reveal little osteopontin immunoreactivity in intimal smooth muscle cells (C, Case 4, ×100). Osteopontin is cytoplasmic in macrophages/foam cells (arrow) and vascular smooth cells (small arrow) in the intima and neointima (D-F, Case 1 and 3, ×400 and ×1,000).

to be found predominantly in lesion macrophages/foam cells but also in vascular smooth muscle cells. As shown in the present study, OPN might have a regulatory role in the vascular calcification by remodeling of extracellular matrix. Non-calcified plaques intervening calcified coronary segments had abundant vasa vasorum within plaque itself, which might induce plaque rupture-hemorrhage with increasing cardiovascular events.

Occlusive coronary artery disease (CAD) is a major cause of death (38). It is well known that atherosclerosis begins early in life and typically progresses silently until the clinical symptoms occur late in the disease (39). Early detection of asymptomatic coronary atherosclerosis has been hindered by the lack of sensitive and specific diagnostic tests (40, 41). The vast majority of significant coronary stenosis are calcified (4, 42), and a high degree of correlation exists between severity of CAD and calcification of the vessel wall (43-47). Atherosclerotic calcification is an organized, regulated process similar to bone formation that occurs only when other factors of atherosclerosis are also present. Although the process of calcification is not completely understood (48), calcified lesions found in coronary arteries have been considered for many years to be the markers of advanced atherosclerotic disease (42). However, several studies (2, 11, 15) have recently shown that intramural calcium deposition may occur early in atherosclerosis, after which it proceeds together with atherosclerotic plaque development.

Recent 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 bone, have been detected in calcified atherosclerotic lesions (49). In atherosclerosis, intimal macrophages and vascular smooth muscle cells express both collagenous (collagen types I and IV) and noncollagenous bone matrix proteins (matrix gamma carboxyglutamate protein, OPN, osteonectin, osteoglycin, and bone morphogenic protein). The association of intimal calcification with lipid, apoptotic cells, and matrix vesicles suggests that the intimal calcification is an active rather than a degenerative process (50-55). Although the biochemical sequence of events leading to atherosclerotic calcification is not well understood, recent attention has

been given to a unique class of proteins known as matrix gamma carboxyglutamate protein (MGP), which has a very high affinity for hydroxyapatite and is actively involved in the transport of calcium out of vessel wall, and the localization in advanced atherosclerotic lesions (17, 56-60). When gamma-carboxylation of glutamic acid residues induces by vitamin K-dependent carboxylase, osteocalcin binds avidly to bone mineral (1, 18, 21). A related protein, MGP is also believed to inhibit mineralization (61, 62). Atherosclerotic processes inhibit the synthesis and/or activity of gamma-glutamate carboxylase, thus perhaps explaining why atherosclerotic arteries contain only about 30% of the carboxylase activity found in normal arterial segments (63).

OPN was identified as one of the matrix proteins associated with mineralization in atherosclerotic coronary artery specimens and was named for its function as a bridge between cells and mineral (1-2, 11, 14). Forming a proteinaceous coating over the solid crystal surface, OPN mediates the attachment of both osteoblasts and osteoclasts to bone mineral through interaction of its highly conserved GRGDS sequence with intergrins (12-13, 19). The major source of OPN protein in both lesions is infiltrating macrophages that are intimately associated with the calcified deposits, although smooth muscle and endothelial cells also synthesize OPN (1, 2, 11, 14-18). As the association with smooth muscle cells mediated vascular calcification, vascular smooth muscle cells expressed the smooth muscle-specific gene SM22α and high levels of MGP but little OPN mRNA (23, 25). These studies suggested that calcifying vascular cells, which may differentiate into osteoblastic cells via several factors, and soluble OPN released near sites of vascular calcification may represent an adaptive mechanism aimed at preventing vascular calcification (23).

Clinically, supplement of vitamin D and calcium is widely used for the treatment of osteoporosis, especially in the elderly. However, pharmacological doses of 1,25 $(OH)_2D_3$ may stimulate vascular calcification through a direct action on vascular smooth muscle cells and the expression of OPN gene, and also decreased secretion of parathyroid hormone-related peptide (64). Furthermore, local production of 1,25$(OH)_2D_3$ by 1a-hydroxylase expressed in macrophages accumulated in atherosclerotic lesions and is also well known to be a potent stimulator of osteoblastic differentiation of vascular cells (65). As described above, the synergy between warfarin and vitamin D is probably explained by hypothesis that warfarin inhibits the activity of MGP as a calcification inhibitor (61, 62). It is important to determine whether long-term supplementation of vitamin D for osteoporosis adjunction with vitamin K-dependent anticoagulant exacerbates vascular calcification and to further clarify the precise roles of noncollagenous bone matrix protein and vascular calcification in order to prevent vascular calcification and other events involved.

Prior pathologic, angiographic and intravascular ultrasound studies have not specifically examined calcium and quantitative definition of disease in the identical arterial segments (26-30). A growing number of investigations (3, 31, 32) has shown the feasibility of using EBCT scanning for detecting and quantifying the coronary artery calcification in in vivo studies. While these studies suggest a general relationship between calcification and the severity of angiographic disease, the interpretation of these scans in predicting luminal narrowing remains unclear (28, 66, 67). The lack of good predictive value between calcification and angiography may be due to vascular remodeling, where arteries increase in size to compensate for the atherosclerotic plaque growth and to preserve the lumen size (68). Micro-CT is a novel and powerful technique that permits the assessment of the three-dimensional pattern of vascular structure and provides a useful means for the study of the spatial distribution of calcification within the vessel wall, such as geometric characterization between calcific and non-calcific segments with intimal neovascularization (33-37, 69-71). This information will give the pathophysiologic understandings of vascular events in the patients with calcified coronary artery disease (71).

As shown in the present study, OPN might have a regulatory role in vascular calcification with extracellular matrix remodeling that may suggest more plaque complication and vascular events.

REFERENCES

1. O'Brien KD, Kuusisto J, Reichenbach DD, Ferguson M, Giachelli C, Alpers CE, Otto CM. *Osteopontin is expressed in human aortic valvular lesions. Circulation 1995; 92: 2163-8.*
2. Bostrom K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL. *Bone morphogenetic protein expression in human atherosclerotic lesions. J Clin Invest 1993; 91: 1800-9.*
3. Agatston A, Janowitz W, Hildner FJ, Zusmer N, Viamonte M, Detrano R. *Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol 1990; 15: 827-32.*
4. Honye J, Mahon DJ, Jain A, White CJ, Ramee SR, Wallis JB, Al-Zarka A, Tobis JM. *Morphological effects of coronary balloon angioplasty in vivo assessed by intravascular ultrasound imaging. Circulation 1992; 85: 1012-25.*
5. Bini A, Mann KG, Kudryk BJ, Shoen FJ. *Noncollagenous bone matrix proteins, calcification, and thrombosis in carotid artery atherosclerosis. Arterioscler Thromb Vasc Biol 1999; 19: 1852-61.*

6. Arad Y, Spadaro L, Goodman K, Lledo-Perez A, Sherman S, Lerner G, Guerci A. *Predictive value of electron beam computed tomography of the coronary arteries. Circulation 1996; 93: 1951-3.*
7. Margolis J, Chen J, Kong Y, Peter R, Behar V, Kisslo J. *The diagnostic and prognostic significance of coronary artery calcification: a report of 800 cases. Radiology 1980; 137: 609-16.*
8. Fitzgerald PJ, Ports TA, Yock PG. *Contribution of localized calcium deposits to dissection after angioplasty: an observational study using intravascular ultrasound. Circulation 1992; 86: 64-70.*
9. Puentes G, Detrano R, Tang W, Wong N, French W, Narahara K, Brundage B, Baksheshi H. *Estimation of coronary calcium mass using electron beam computed tomography: a promising approach for predicting coronary events? Circulation 1995; 92(Suppl): I-313.*
10. Simmons DB, Schwartz RS, Edwards WD, Sheedy PF, Breen JF, Rumberger JA. *Noninvasive definition of anatomic coronary disease by ultrafast computed tomographic scanning: a quantitative pathologic comparison study. J Am Coll Cardiol 1992; 20: 1118-26.*
11. Fitzpatrick LA, Severson A, Edwards WD, Ingram RT. *Diffuse calcification in human coronary arteries: association of osteopontin with atherosclerosis. J Clin Invest 1994; 94: 1597-604.*
12. Mckee MD, Nanci A. *Osteopontin at mineralized tissue interfaces in bone, teeth, and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover, and repair. Microsc Res Tech 1996; 33: 141-64.*
13. Hoyer JR, Otvos L Jr, Urge L. *Osteopontin in urinary stone formation. Ann N Y Acad Sci 1995; 760: 257-65.*
14. Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM. *Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest 1993; 92: 1686-96.*
15. Hirota S, Imakita M, Kohri K, Ito A, Morii E, Adachi S, Kim HM, Kitamura Y, Yutani Y, Nomura S. *Expression of osteopontin messenger RNA by macrophages in atherosclerotic plaques; a possible association with calcification. Am J Pathol 1993; 143: 1003-8.*
16. Ikeda T, Shirasawa T, Esaki Y, Yoshiki S, Hirokawa K. *Osteopontin mRNA is expressed by smooth muscle-derived foam cells in human atherosclerotic lesions of the aorta. J Clin Invest 1993; 92: 2814-20.*
17. Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL. *High expression of genes for calcification-regulation proteins in human atherosclerotic plaques. J Clin Invest 1994; 93: 2393-402.*
18. O'Brien ER, Garvin MR, Stewart DK, Hinohara T, Simpson JB, Schwartz SM, Giachelli CM. *Osteopontin is synthesized by macrophages, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques. Arterioscler Thromb Vasc Biol 1994; 14: 1648-56.*
19. Butler WT, Ridall AL, McKee MD. *Osteopontin. In: Bilezikian JP, Raiaz LG, Rodan GA, eds. Principles of bone biology. San Diego: Academic Press, 1996; 167-81.*
20. Hunter GK, Kyle CL, Goldberg HA. *Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J 1994; 300: 723-8.*
21. Boskey AL, Maresca M, Ullrich W, Doty SB, Butler WT, Prince CW. *Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite formation and growth in a gelatin-gel. Bone Miner 1993; 22: 147-59.*
22. Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresh DA. *Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin avb3 potentiate bone resorption. J Biol Chem 1993; 268: 9901-7.*
23. Wada T, McKee MD, Steitz S, Giachelli CM. *Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. Circ Res 1999; 84: 166-78.*
24. Li G, Bakir SE, Oparil S, Thompson JA. *Estrogen attenuates osteopontin production by activated smooth muscle cells: a potential mechanism of estrogen-induced vasoprotection. Circulation 2000; 35: 295A.*
25. Shanahan CM, Cary NR, Salisbury JR, Proudfoot D, Weissberg PL, Edmonds ME. *Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification. Circulation 1999; 100: 2168-76.*
26. Rumberger JA, Simons DB, Fitzpatrick LA, Sheedy PF, Schwartz RS. *Coronary artery calcium area by electron beam computed tomography and coronary atherosclerotic plaque area: a histopathologic correlative study. Circulation 1995; 92: 2157-62.*
27. Mautner GC, Mautner SL, Froelich J, Feuerstein IM, Proschan MA, Roberts WC, Doppman JL. *Coronary artery calcification: assessment with electron beam CT and histomorphometric correlations. Radiology 1995; 192: 619-23.*
28. Rumberger JA, Sheedy PFI, Breen JF, Schwartz RS. *Coronary calcium, as determined by electron beam computed tomography, and coronary disease on arteriogram: effect of patient's sex on diagnosis. Circulation 1995; 91: 1363-7.*
29. Rumberger JA, Sheedy PF, Breen JF, Schwartz RS. *Electron beam computed tomographic coronary calcium area score cutpoints and severity of associated angiographic lumen stenosis. J Am Coll Cardiol 1997; 29: 1542-8.*
30. Baumgart D, Schmermund A, Goerge G, Haude M, Ge J, Adamzik M, Sehnert C, Altmaier K, Groenemeyer D, Seibel R, Erber R. *Comparison of electron beam computed tomography with intracoronary ultrasound and coronary angiography for detection of coronary atherosclerosis. J Am Coll Cardiol 1997; 30: 57-64.*
31. Reinmuller R, Lipton MJ. *Detection of coronary artery calcification by computed tomography. Dynamic Cardiovascular Imaging 1987; 1: 139-45.*
32. Tanenbaum SM, Kondos GT, Veselik KE, Prendergast MR,

Brundage BH, Chomka EV. *Detection of calcific deposits in coronary arteries by ultrafast computed tomography and correlation with angiography. Am J Cardiol 1989; 63: 870-2.*
33. Flannery BP, Dickman HW, Roberge WG, D'Amico KL. *Three-dimensional x-ray micro tomography. Science 1987; 237: 1439-44.*
34. Avula RTV, Dunsmuir JH, Beighley PE, Thomas PJ, Faridani A, Ritman EL. *A micro tomographic technique enhanced by novel image reconstruction algorithm: application to rat coronary vessels. FASEB J 1994; 8: A854.*
35. Ritman EL, Dunsmuir JH, Faridani A, Finch DV, Smith KT, Thomas PJ. *Local reconstruction applied to x-ray tomography. In: Chavent G, Papanicolaou G, Sack P, Symes W, eds. IMA volumes in mathematics and its applications: inverse problems in wave propagation. New York: Springer-Verlag, 1997; 443-52.*
36. Chilian WM. *Coronary microcirculation in health and disease: summary of an NHLBI workshop. Circulation 1997; 95: 522-8.*
37. Kwon HM, Sangiorgi G, Ritman EL, McKenna C, Holmes DR, Schwartz RS, Lerman A. *Enhanced coronary vasa vasorum Neovascularization in experimental hypercholesterolemia. J Clin Invest 1998; 101: 1551-6.*
38. Friedewald ST. *Epidemiology of cardiovascular disease. In: Wyngaarden JB, Smith LH, eds. Cecil's textbook of medicine. Philadelphia: W.B. Saunders, 1988; 179-83.*
39. Rose G. *ABC of vascular diseases. Epidemiology of atherosclerosis. BMJ 1991; 303: 1537-9.*
40. Wald NJ. *Cholesterol and coronary heart disease: to screen or not to screen. In: Marmot M, Elliott P, eds. Coronary heart disease epidemiology. Oxford: Oxford University Press, 1992; 12-24.*
41. Subcommittee on Exercise Testing. *Report of the American College of Cardiology/American Heart Association Task Force on Assessment of Cardiovascular Procedures: guidelines for exercise testing. J Am Coll Cardiol 1986; 8: 725-38.*
42. Blankenhorn DH. *Coronary calcification: a review. Am J Med Sci 1961; 242: 1-9.*
43. Blankenhorn DH. *Relationship between calcium content of early and moderately advanced atheromas and diffuse aortic calcification. J Med Sci 1961; 242: 1-10.*
44. Beadenkopf WG, Daoud AS, Love BM. *Calcification in the coronary arteries and its relationship to arteriosclerosis and myocardial infarction. Am J Roentgenol 1964; 92: 865-71.*
45. Eggen DA, Strong JP, McGill HC Jr. *Coronary calcification. Relationship to clinical significant coronary lesion and race, sex and topographic distribution. Circulation 1965; 32: 948-55.*
46. Tampas JP, Soule AB. *Coronary arterial calcification. Its incidence and significance in patients over forty years of age. Am J Roentgenol 1966; 97: 369-76.*
47. Frink AJ, Achor RW, Brown AL Jr, Kincaid OW, Branderburg RO. *Significance of calcification of the coronary arteries. Am J Cardiol 1970; 26: 241-7.*
48. Eisenstein R, Trueheart RE, Hass GM. *Calcification in biology systems. In: Sognanes R, ed. American association for the Advancement of Science. Washington D.C.: 1960; 64: 281-305.*
49. Kim KM. *Calcification of matrix vesicles in human aortic valve and aortic media. Fed Proc 1976; 35: 156-62.*
50. Doherty TM, Detrano RC. *Coronary arterial calcification as an active process: a new perspective on an old problem. Calcif Tissue Int 1994; 54: 224-30.*
51. Demer LL. *A skeleton in the atherosclerosis closet. Circulation 1995; 92: 2029-32.*
52. Proudfoot D, Shanahan CM, Weissberg PL. *Vascular calcification: new insights into an old problem. J Pathol 1998; 185: 1-3.*
53. Isner JM, Kearney M, Bortman S, Passeri J. *Apoptosis in human atherosclerosis and restenosis. Circulation 1995; 91: 2703-11.*
54. Tanimura A, McGregor DH, Anderson HC. *Matrix vesicles in atherosclerotic calcification. Proc Soc Exp Biol Med 1983; 172: 173-7.*
55. Hirsch D, Azoury R, Sarig S, Kruth HS. *Colocalization of cholesterol and hydroxyapatite in human atherosclerotic lesions. Calcif Tissue Int 1993; 52: 94-8.*
56. Vermeer C. *Gamma-carboxyglutamate-containing proteins and the vitamin K-dependent carboxylase. Biochem J 1990; 266: 625-36.*
57. Price PA. *Gla-containing proteins of bone. Connect Tissue Res 1989; 21: 51-60.*
58. Cotran RS, Kumar V, Robbins SL. *Cellular injury and cellular death. In: Robbins SL, ed. Pathological basis of disease. 5th ed. Philadelphia: W.B. Saunders, 1994; 1-35.*
59. Fleet JC, Hock JM. *Identification of osteocalcin mRNA in nonosteoid tissue of rats and humans by reverse transcription-polymerase chain reaction. J Bone Miner Res 1994; 9: 1565-73.*
60. Levy RJ, Howard SL, Oshry LJ. *Carboxyglutamic acid (Gla) containing proteins of human calcified atherosclerotic plaque solubilized by EDTA. Molecular weight distribution and relationship to osteocalcin. Atherosclerosis 1986; 59: 155-60.*
61. Price PA, Faus SA, Williamson MK. *Warfarin-induced artery calcification is accelerated by growth and vitamin D. Arterioscler Thromb Vasc Biol 2000; 20: 317-27.*
62. Price PA, Faus SA, Williamson MK. *Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. Arterioscler Thromb Vasc Biol 1998; 18: 1400-7.*
63. de Boer-van den Berg MA, Van Haarlem LJM, Vermeer C. *Vitamin K-dependent carboxylase in human vessel wall. Thromb Res 1986; S6: 134.*
64. Jono S, Nishizawa Y, Shioi A, Morri H. *1,25-Dihydroxyvitamin D3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. Circulation 1998; 98: 1302-6.*
65. Adams JS. *Extrarenal production and action of active vitamin D metabolites in human lymphoproliferative diseases. In: Feldman D, Glorieux FH, Pike W, eds. Vitamin D. New York:*

Academic Press, 1997; 903-21.

66. Breen JF, Sheedy PF, Schwartz RS, Stanson AW, Kaufmann RB, Moll PP, Rumberger JA. *Coronary calcification detected with fast-CT as a marker of coronary artery disease: works in progress. Radiology 1992; 185: 435-9.*
67. Kaufmann RB, Peyser PA, Sheedy PF, Rumberger JA, Schwartz RS. *Quantification of coronary artery calcium by electron beam computed tomography for determination of severity of angiographic coronary disease in younger patients. J Am Coll Cardiol 1995; 25: 626-32.*
68. Glagov S, Weisenberg BA, Zarins CK, Staunkunavicius R, Koletties GJ. *Compensatory enlargement of human atherosclerotic coronary arteries. N Engl J Med 1987; 316: 1371-5.*
69. Barger AC, Beeuwkes R III, Lainey LL, Silverman KJ. *Hypothesis: vasa vasorum and neovascularization of human coronary arteries: a possible role in the pathophysiology of atherosclerosis. N Engl J Med 1984; 310: 175-7.*
70. Kumamoto M, Nakashima Y, Sueishi K. *Intimal neovascularization in human coronary atherosclerosis: its origin and pathophysiological significance. Hum Pathol 1995; 26: 450-6.*
71. Barger AC, Beeuwkes R III. *Rupture of coronary vasa vasorum as a trigger of acute myocardial infarction. Am J Cardiol 1990; 66: 41-3G.*