Diffuse Calcification in Human Coronary Arteries

Association of Osteopontin with Atherosclerosis

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Abstract

Coronary atherosclerosis is frequently associated with calcification of arterial plaque. To understand the mechanisms responsible for the formation of atherosclerotic calcification, we examined human coronary arteries for the presence and extent of mineral. In sections stained specifically for mineral, staining was diffuse and present in all atherosclerotic plaques. Hydroxyapatite was not detected in normal coronary artery sections. Distribution of hydroxyapatite coincided with a similar distribution of calcium detected by a radiodense pattern using contact microradiography of the same sections before cytochemical staining. By energy-dispersive x-ray microanalysis, the chemical composition of calcified sites was identical to hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$), the major inorganic component of bone. Osteopontin is a phosphorylated glycoprotein with known involvement in the formation and calcification of bone and is regulated by local cytokines. Human coronary artery segments (14 normal and 34 atherosclerotic) obtained at autopsy were evaluated immunohistochemically using polyclonal antibodies generated against human osteopontin. Immunohistochemistry for osteopontin indicated intense, highly specific staining in the outer margins of all diseased segments at each calcification front; staining was evident throughout the entire plaque. Conversely, arterial segments free of atheroma and calcification and sections treated with nonimmune serum had no evidence of positive staining. Osteopontin, a protein involved in mineralization is specifically associated with calcific coronary atheroma and may play an important role in the onset and progression of this disease in human coronary arteries. The deposition of noncollagenous proteins such as osteopontin may regulate the presence or absence of calcification and ultimately alter vessel compliance. (*J. Clin. Invest.* 1994. 94:1597–1604.) Key words: calcification • atherosclerosis • osteopontin • mineralization • plaque

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Introduction

Coronary atherosclerosis begins early in life and progresses slowly until clinically manifested as symptomatic angina pectoris or sudden plaque rupture with thrombosis, vessel occlusion, and myocardial infarction with or without sudden death (1–3). The occurrence of coronary artery calcification is common in patients with known coronary artery disease and increases dramatically as a function of age (4, 5; for review see reference 6). In a study of 65 autopsy derived hearts from patients over 60 years of age, 94% of coronary arteries had some degree of calcification (7).

The onset and progression of calcification in arterial plaques is poorly understood. However, accumulating evidence suggests that pathologic calcification of atherosclerotic vessels shares features with normal bone such as cellular proliferation, matrix deposition, and calcification. Type I collagen is associated with bone formation and is the principle collagen found in atherosclerotic plaques (8, 9). Another common feature of bone and calcified atherosclerotic arteries is the presence of phosphatases and calcium binding phospholipids in matrix vesicles that serve as nucleators of crystal formation (10–13). Early work provided evidence that mineral deposits in arterial plaques consist of crystalline hydroxyapatite, the major inorganic component of bone (14, 15). Although the chemical composition of calcium within mineral deposits in human atherosclerotic plaques have been defined (15, 16) and the radiographic appearance of calcific plaques described (17), no study has addressed the extent and distribution of calcification within undecalcified atherosclerotic arteries at a cellular level. In this study, we use methodology unique to the preservation of skeletal tissue to determine the presence of calcification within coronary arteries.

Osteopontin, a noncollagenous protein associated with bone formation and mineralization, avidly binds calcium and hydroxyapatite and can be detected in bone using immunocytochemical techniques (18–20). This phosphoprotein is present in high concentration at the mineralization front of bone matrix (21). To understand the mechanism of calcification in human coronary atherosclerosis we examined normal and calcified atherosclerotic coronary arteries for the presence of osteopontin using immunocytochemistry and evaluated the extent of and type of mineralization.

Methods

Tissue preparation. Coronary arteries were obtained at autopsy from seven patients with known coronary artery disease. Patients with metabolic bone disease, disorders of calcium homeostasis, malignancy, or ingesting medications known to affect calcium metabolism (glucocorticoids, etidronate, vitamin D, fluoride) were excluded. Patients ranged in age from 44 to 81. Specimens were fixed in 4% paraformaldehyde, decalcified in formic acid and embedded in paraffin. Coronary artery segments (14 normal and 34 atherosclerotic) were sectioned (5 μm)

and immunostained as described below. To study undecalcified sections, the following method of tissue preservation was used. Separate specimens were fixed in ethanol and dehydrated in ascending alcohols and embedded (undecalcified) in glycolmethylmethacrylate (GMA)[1] using a temperature-controlled method (Rainier Technical Products, Seattle, WA). Briefly, arteries were infiltrated for 3 d in a mixture of the following: 81% (vol/vol) uninhibited methylmethacrylate, 8% (wt/vol) polyethylene glycol disterate (1540), 6.5% (vol/vol) 2-hydroxyethyl methacrylate, 4% dibutylphthalate, and 0.65% benzoyl peroxide. Infiltrated biopsies were placed in fresh monomer containing accelerator and allowed to polymerize onto aluminum chucks at room temperature in the presence of nitrogen. Unmounted sections (5 μm) were stained as described below.

Polyclonal antiserum raised in a rabbit (LF-7) was generated against purified human osteopontin (22) and kindly provided by Dr. Larry Fisher (National Institute of Dental Research, National Institutes of Health, Bethesda, MD).

Immunohistology. Paraffin was removed from the tissue sections using xylene (100%). Tissue was rehydrated in descending ethanols and blocked in Tris-buffered saline (TBS, 0.05 M Tris, 0.01% bovine serum albumin, 0.9% NaCl, pH 7.5) containing 0.3% casein and 10% normal goat serum. Sections were stained using Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations, with modification. Incubations with primary and secondary antibody (biotinylated goat anti–rabbit) were performed at room temperature followed by two 15-min washes in TBS containing 0.02% Triton X-100. Endogenous peroxidase activity was inhibited with 1.5% H_2O_2 and 0.1% sodium azide in 50% methanol for 15 min. Bound secondary antibody was detected with peroxidase-conjugated avidin–biotin complex and visualized using 0.05% diaminobenzidine and 0.01% H_2O_2. Sections were rinsed with tap water, dehydrated with ascending alcohols and cleared with xylene. Control sections were stained using normal rabbit serum at the same dilution as primary antibody.

Energy-dispersive x-ray microanalysis. To determine the chemical composition of calcified plaques, 20-μm sections of GMA-embedded atherosclerotic arteries were analyzed by energy-dispersive x-ray microanalysis. Sections of undecalcified artery were mounted on aluminum stubs with colloidal graphite. 10 spectra were acquired for each sample using a live count time of 120 s. Calcium to phosphorus molar ratios were compared with a known standard of hydroxyapatite (Sigma Chemical Co., St. Louis, MO).

Contact microradiography. To determine the extent of calcification in atherosclerotic arteries, sections of GMA-embedded specimens were cut to 100 μm using an Isomet saw and placed on emulsion-coated slides (Eastman Kodak Co., Rochester, NY) for microradiography. Sections were exposed to x-ray (20 kV) for 5 min and developed according to the manufacturers' recommendations.

Staining. Sections of plastic-embedded coronary arteries were stained with Von Kossa staining method (specific for phosphate) and counter stained with van Gieson and aldehyde fuschin. Alternately, sections were stained with Goldner's-Masson-Trichrome. Paraffin-embedded sections were stained with hematoxylin and eosin and Lawson's elastic-tissue van Gieson using standard procedures (23). Sections stained using normal serum (non-immune controls) revealed no evidence of nonspecific staining.

Results

Histologic staining and microradiography. Paraffin embedded sections of decalcified atherosclerotic coronary arteries were stained for hematoxylin and eosin (Fig. 1 *A*). Fig. 1 *B* represents a section stained with Goldner's-Masson-Trichrome. Hydroxyapatite is represented by the blue-green color on this histologic section. Uncalcified tissue appears pink or red. In areas of human coronary arteries that were not associated with atherosclerotic plaque, there was a marked absence of hydroxyapatite as detected with Goldner's-Masson-Trichrome stain (Fig. 2). The amount and extent of calcification was variable; however, it was more extensive than anticipated. Frequently, the calcification appeared diffuse and interstitial (Fig. 3, *A–C*), and in other sections, an abrupt transition from uncalcified to calcified sections was noted (Fig. 3 *D*). Extent of phosphate deposition was confirmed by use of the von Kossa stain and revealed patterns of distribution identical to the mineral detected with Goldner's-Masson-Trichrome (data not shown).

To determine the extent of calcification and to confirm the nature of the calcification in these atherosclerotic arteries, separate undecalcified specimens were embedded in GMA and 100-μm sections subjected to x-ray microradiography. Deposits of mineral were apparent as radiodense images in the microradiograph (Fig. 4 *B*) and compared to von Kossa (data not shown) or Goldner's-Masson-Trichrome staining of the same sections (Fig. 4 *A*). Based on these analyses, atherosclerotic coronary arteries revealed extensive, diffuse calcification. Although the extent of calcification varied, areas of mineralization were localized to the intima and media of vessel walls and extended into the adventitia. Diffuse deposits were often observed throughout the media (Figs. 1, 3, and 4).

Energy-dispersive x-ray microanalysis. To assess the mineral composition of calcified plaques, sections of coronary arteries were analyzed by energy-dispersive x-ray microanalysis. Calcified plaques contained a calcium to phosphate molar ratio of 1.55:1 to 1.70:1 which corresponds closely to the known ratio of 1.66:1 of hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$), the major inorganic component of bone (Fig. 5). Normal vessel segments and segments contiguous to atherosclerotic sections but free of plaques did not display profiles consistent with the presence of hydroxyapatite, thus confirming the absence of mineral in normal vessels.

Immunohistochemical staining of osteopontin in coronary arteries. To determine whether calcification was associated with osteopontin, a matrix protein involved in normal bone mineralization, coronary arteries were immunostained using polyclonal antibodies previously shown to detect osteopontin in sections of human bone (19). Immunocytochemistry revealed intense and highly specific staining. Areas of positive stain were localized primarily to the outer margins of plaques at the calcification front (Fig. 6,*A* and *B*). Less intense, diffuse staining was evident within the central portion of each plaque. Tissue surrounding osteopontin-positive plaques stained negative for osteopontin as did arterial segments free of atheroma (Fig. 7 *B*). Sections stained using normal serum (nonimmune controls) showed no evidence of nonspecific staining for osteopontin (Fig. 7 *A*).

Discussion

Coronary atherosclerosis slowly progresses until clinically manifested as symptomatic angina pectoris or sudden plaque rupture with thrombosis, vessel occlusion, and myocardial infarction. Coronary artery calcification is common in patients with known coronary artery disease and increases dramatically with age (4–7, 24). The onset and progression of calcification in arterial plaque shares features with other calcified tissues such as cellu-

1. *Abbreviation used in this paper:* GMA, glycolmethylmethacrylate.

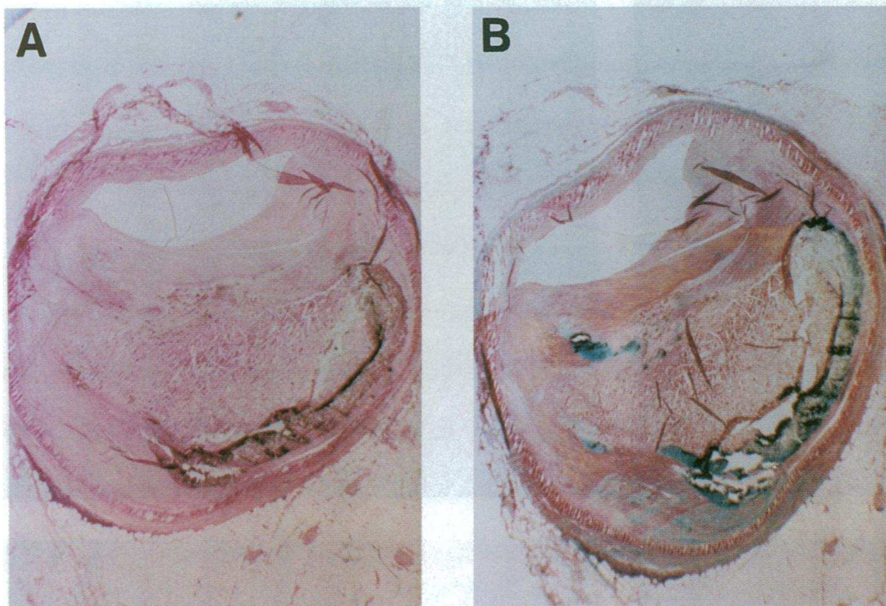

Figure 1. Undecalcified specimens of human coronary artery were obtained at autopsy from seven patients with known coronary artery disease. Specimens were fixed in ethanol, dehydrated in ascending ethanols embedded in glycol-methylmethacrylate (GMA) using a temperature-controlled method as in Methods. Unmounted sections were stained with Goldner's-Masson-Trichrome in which calcified tissue appears blue-green and uncalcified tissue appears red. (*A*) Contiguous section of atherosclerotic plaque stained with hematoxylin and eosin. (*B*) Section of atherosclerotic plaque indicating the large amount of hydroxyapatite present.

lar proliferation, matrix deposition and mineralization. The presence of Type I collagen, phosphatases, calcium binding phospholipids, and crystalline hydroxyapatite and the regulation of these processes by growth factors and cytokines are features common to the normal calcification process and calcification associated with coronary atherosclerosis (6, 8–15, 24, 25).

The cell and matrix composition in atherosclerotic lesions found in young individuals has been reported (24). The vessels in individuals < 40 yr old contain intimal lesions ladened with macrophages, lipid-ladened macrophages (foam cells), and lipid-enriched smooth muscle cells and fatty streaks. As early as the second decade, calcification associated with atherosclerosis can be detected. Studies have suggested that calcification may represent an independent pathologic process in the development of coronary atherosclerosis (3–6, 17). The ability to examine coronary arterial plaque as an undecalcified tissue reveals the large extent that mineralization occurs within the plaque. The diffuse nature of the mineralization (Figs. 1 and 3) and the presence of calcification in the adventitia suggests that this process may play a major role in the alterations in compliance and elasticity that have long been associated with plaque formation.

Due to the concern regarding the specificity of histochemical stains for the presence of mineralization, we performed contact microradiography to confirm the diffuse, interstitial nature of mineral deposition in atheroma (Fig. 4). Energy-dispersive x-ray microanalysis confirmed the chemical composition of calcified substrate within coronary arteries. Normal coronary

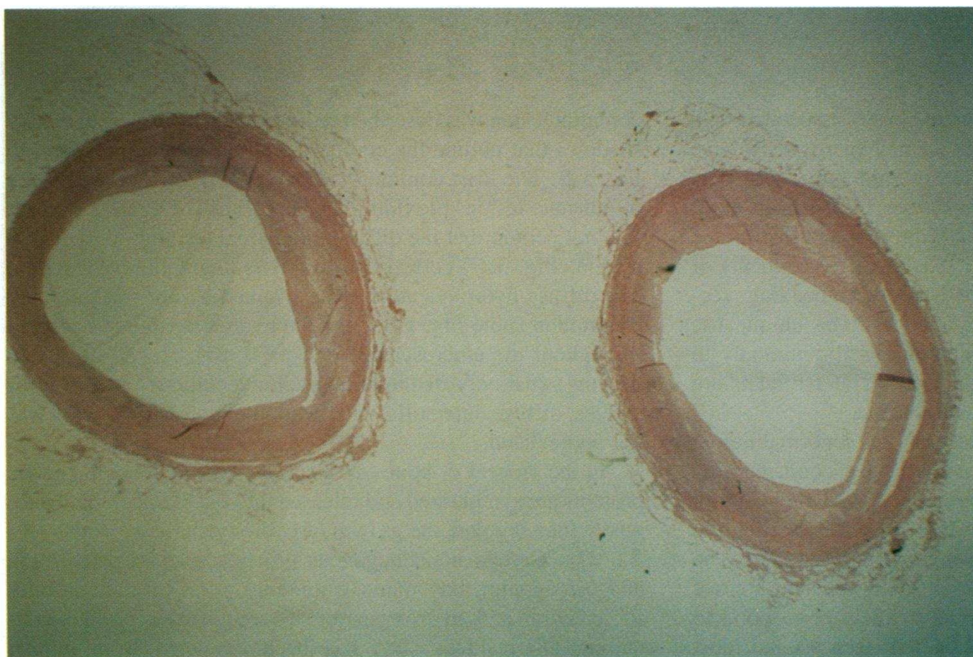

Figure 2. Undecalcified sections of normal human coronary artery. Sections were preserved as indicated in the legend to Fig. 1. Note the lack of blue-green staining that is usually associated with calcified matrix.

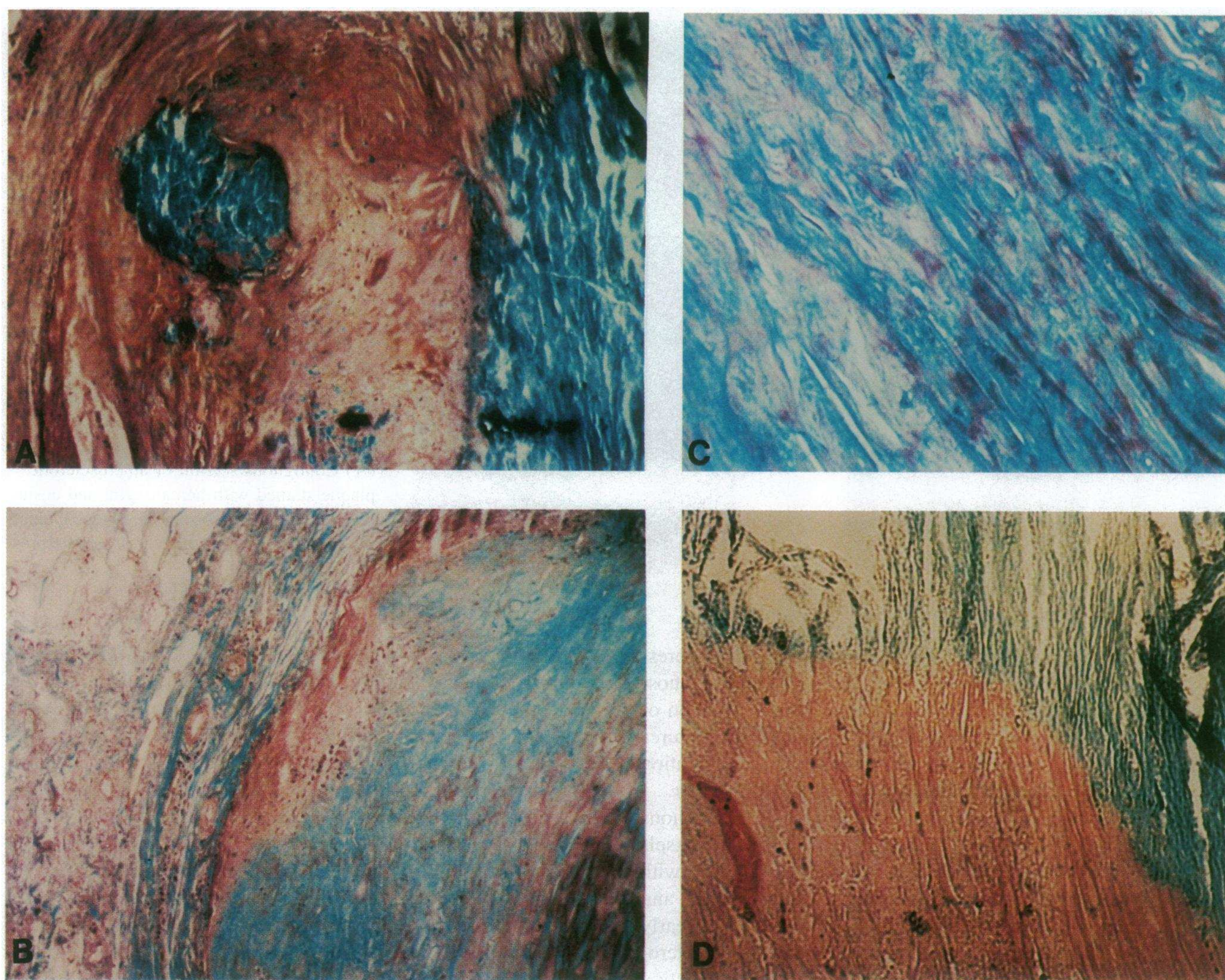

Figure 3. Undecalcified sections of atherosclerotic human coronary artery. (*A*) Large concentration of calcium in center of plaque. (*B*) High power photomicrograph of coronary artery adventitia associated with calcification. (*C*) High power section of atherosclerotic plaque. Note the diffuse interstitial staining of calcified matrix. (*D*) High power photomicrograph of atherosclerotic plaque. Note the abrupt transition from calcified (*blue-green color*) to uncalcified (*pink-red color*) matrix.

arteries and segments of artery that were closely associated with atheromata but free of plaque did not display energy-dispersive profiles consistent with the presence of mineral. These data confirm and extend the recent observations by Boström et al. (15). The calcified matrix is frequently present and distributed widely within each plaque (Fig. 3). Historically, segments of coronary artery have been decalcified before embedding, sectioning, staining and mounting for analysis. The unique use of undecalcified methodology, a technique used commonly to evaluate bone specimens, indicates the diffuse, interstitial nature of calcification within an atheromatous plaque.

Calcific lesions were recognized early as abnormalities in coronary arteries (17). Numerous studies have confirmed the presence of calcium in coronary arteries in association with atherosclerosis. Blankenhorn and Stern defined this distribution of calcium in three major patterns using radiography (26). The most common lesion was the small, discrete punctate areas 1 to 10 mm in diameter. These areas most likely correspond to the large lesion noted in Figs. 1 *B* or 3 *A*. The second most common lesion was described radiographically as dense, blocky shadows that outline the coronary wall and may correspond to Fig. 3 *B*. The least common radiographic patterns was a series of punctate lesions providing the appearance of a railroad track. It is not certain that the diffuse, interstitial staining of hydroxyapatite (Fig. 3 *C*) correspond to this radiographic designation. The diffuse hydroxyapatite as determined by our methodology is common (note Fig. 1 *B*), and the hydroxyapatite is scattered throughout the atherosclerotic plaques. It is likely that due to the low sensitivity of the radiographic technique used in prior studies, diffuse, interstitial calcification of coronary arteries was not appreciated.

In the process of bone calcification, several noncollagenous proteins are synthesized and released into the extracellular space where they regulate the growth of hydroxyapatite crystals (18–22, 27). Certain noncollagenous proteins such as osteocalcin and osteopontin may regulate mineralization by their affinity for calcium and hydroxyapatite. Osteocalcin, a protein containing Gla-residues (28) has been extracted from human

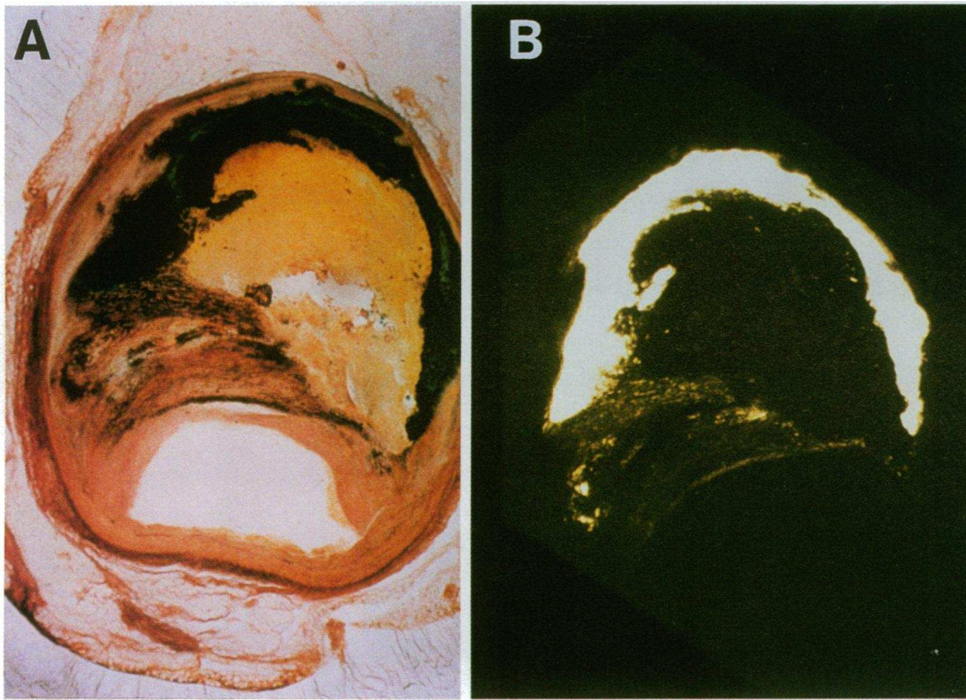

Figure 4. Methylmethacrylate embedded specimens were sectioned and stained with Goldner's-Masson-Trichrome. Blue-green areas represent undecalcified tissue (hydroxyapatite). Red areas represent decalcified tissue. To confirm the nature of the calcification, separate undecalcified specimens were embedded as detailed in Methods, and 100-μm sections were subjected to x-ray microradiography. Deposits of mineral are apparent as radiodense images in the microradiograph (*B*) and compared with staining with Goldner's-Masson-Trichrome (*A*).

calcified arterial plaques (29). An additional calcium-binding Gla-containing protein named plaque Gla protein (PGP) was isolated from human calcified plaque (30). Osteocalcin plays an essential role in normal skeleton development, and gender-related differences in skeletal tissue suggest that this phosphorylated glycoprotein may play an important role in the pathogenesis of several disorders (31). Gamma carboxyglutamate, or Gla, is an amino acid residue with a high affinity for binding hydroxy-apatite. Gijsbers and co-workers have indicated that gamma-glutamate carboxylase activity is increased in normal arteries as compared to atherosclerotic vessels (30). In the normal vessel, therefore, Gla proteins may prevent the deposition of hydroxyapatite into the vessel wall (6); thus the in vivo and in vitro roles for the Gla proteins remain unclear.

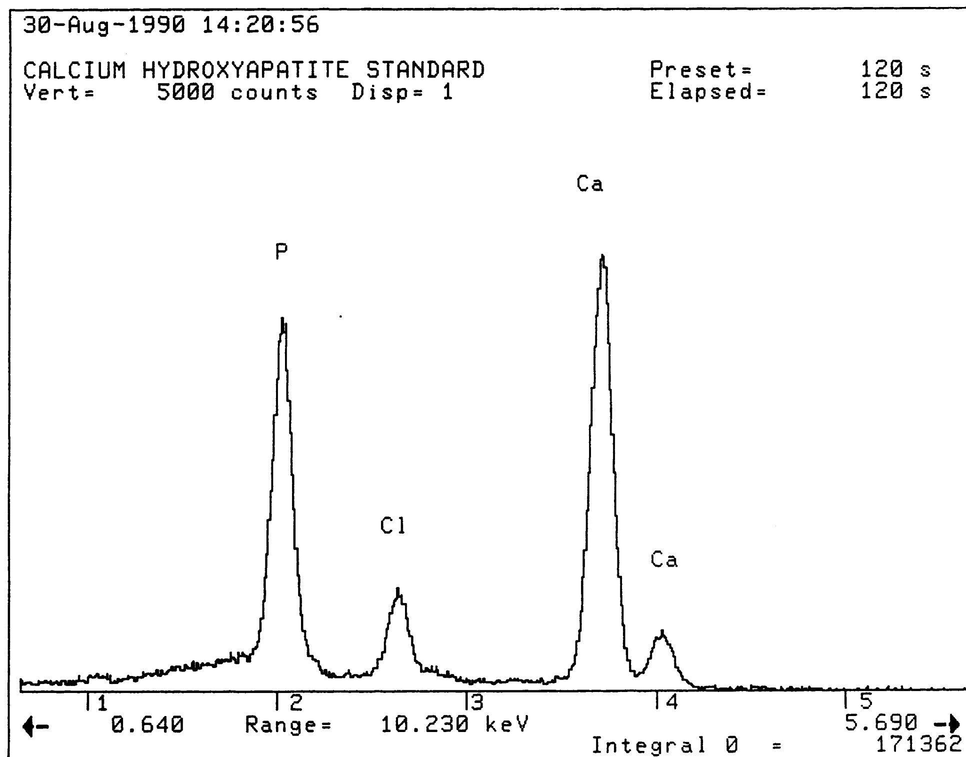

Figure 5. Sections of coronary artery were analyzed by energy-dispersive x-ray microanalysis. Sections of undecalcified coronary artery were mounted on aluminum stubs with colloidal graphite. 10 spectra were acquired for each sample using a live count time of 120 s. Calcium to phosphorus molar ratios were compared with a known standard of hydroxyapatite.

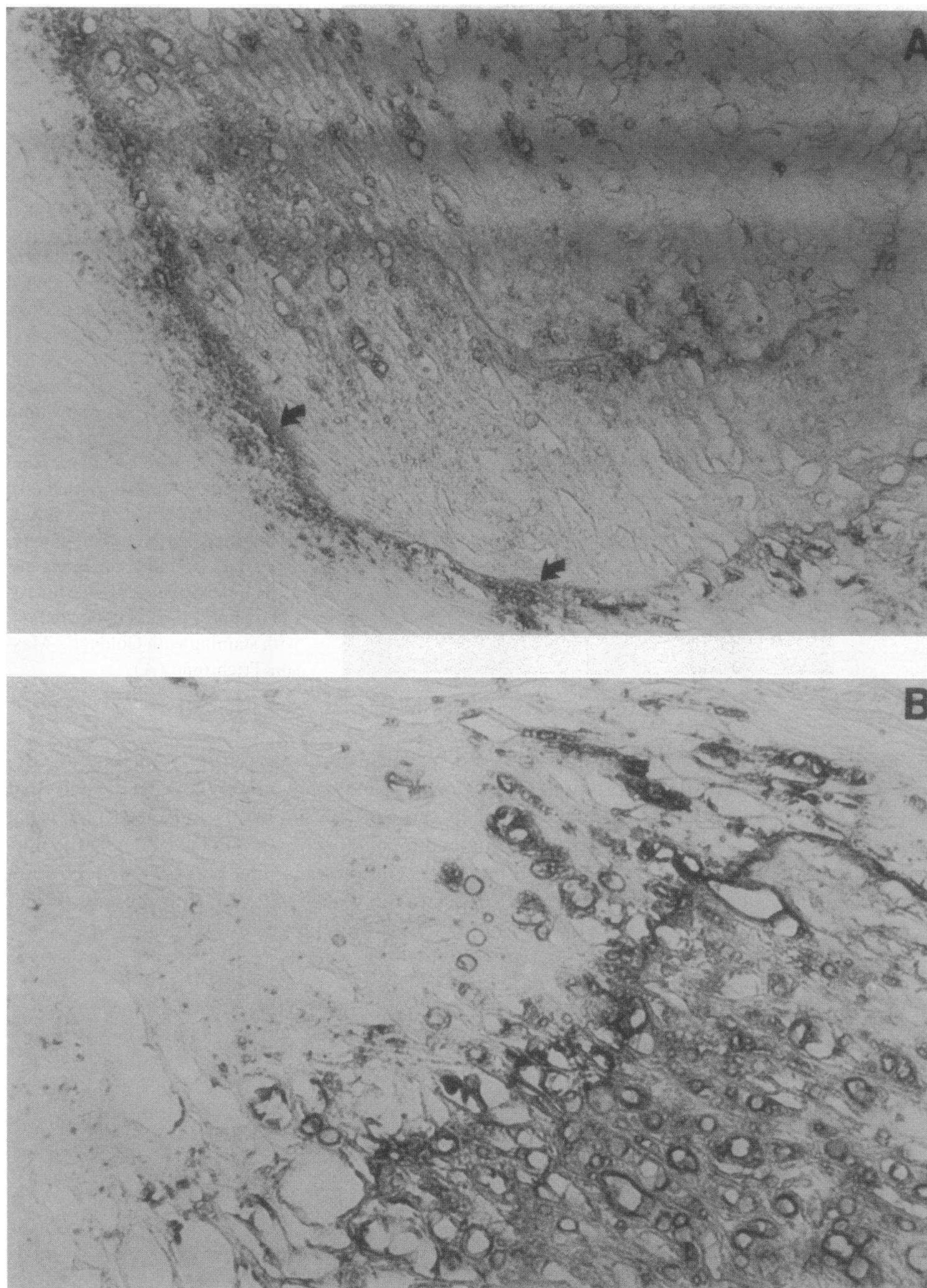

Figure 6. Polyclonal antiserum generated against purified human osteopontin was used to immunostain sections of coronary artery. (*A*) Areas of positive stain were localized primarily to the outer margins of plaques at the calcification front. Less intense staining was evident within the central portion of each plaque magnification ×100. (*B*) Magnification of immunostaining for osteopontin. ×400.

Osteopontin is a complex multifunctional protein associated with mineral binding and cell attachment (for review see reference 32). Osteopontin belongs to a family of unique phosphorylated glycoproteins that contain highly conserved polyaspartic acid- and polyglutamic acid-rich sequences that are common to mineral-binding proteins (21). The presence of osteopontin at the mineralization front in human atherosclerotic coronary arteries provides evidence for the role of the protein in the pathogenesis of calcified plaque. In vitro, this phosphoprotein stimulates (33) or inhibits (34) crystal formation depending on the concentration of osteopontin and on whether mineralization was initiated. Accordingly, it is uncertain whether osteopontin localized to the mineralization front of calcified plaques in atherosclerotic coronary arteries may act to promote calcification or bind these locations in an attempt to reduce the rate of calcification. Our finding of osteopontin in atherosclerotic human coronary arteries and its absence in normal human coronary arteries supports the report describing increased expression of osteopontin in rat arteries after balloon injury (35). In addition, these investigators demonstrated the presence of osteopontin in human atherosclerotic plaques and suggest a role in the mediation of arterial neointimal formation. Osteopontin is present in high concentrations in the mineralization front of rat and chicken bone (21). Our findings of a similar pattern of distribution of osteopontin between bone and atherosclerotic vessels supports the hypothesis that calcification of bone and arteries share common mechanisms. The production and/or accumulation of matrix proteins in atherosclerotic coronary arteries that are nor-

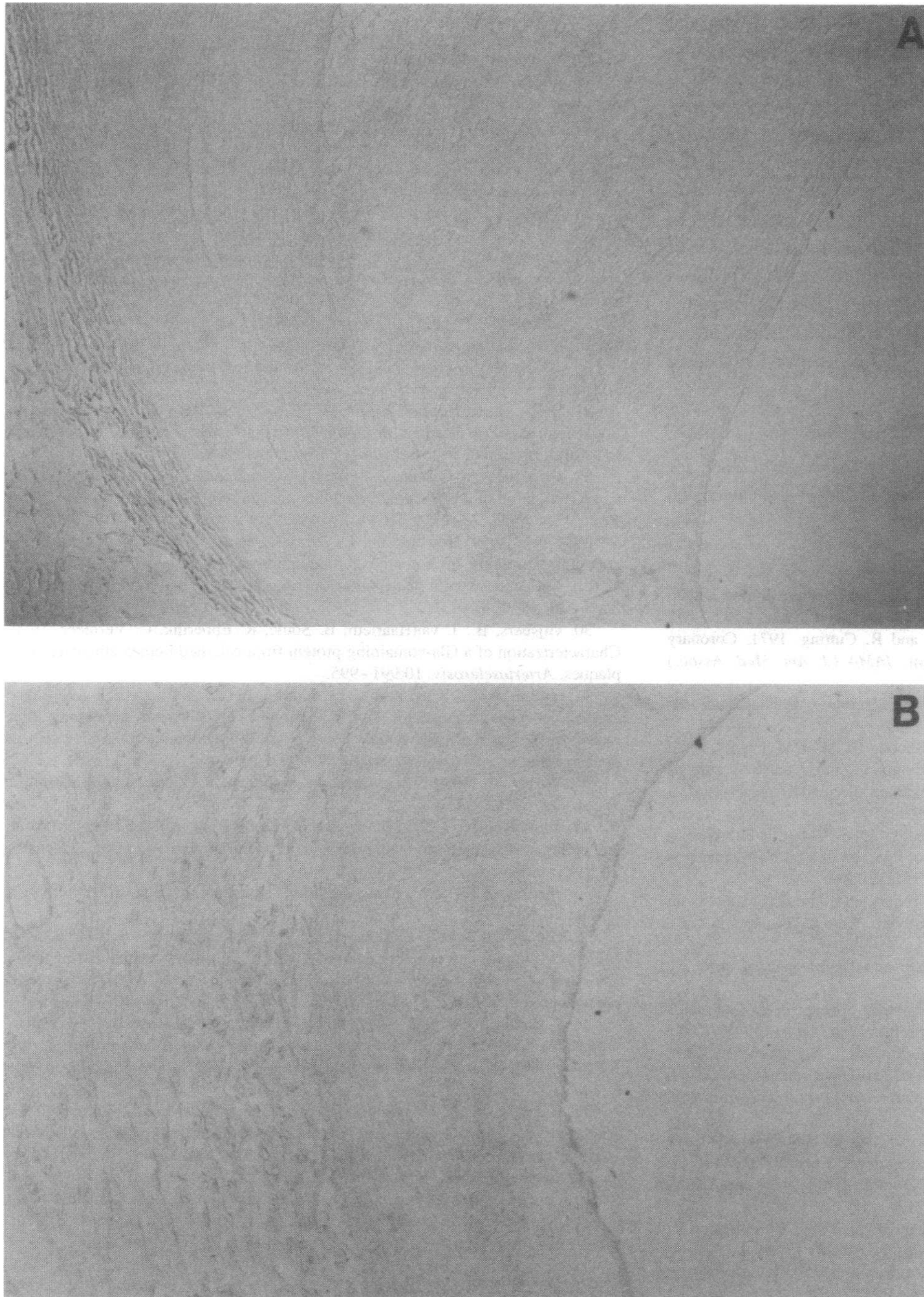

Figure 7. (*A*) Section of atherosclerotic plaque stained with normal rabbit serum. No staining for osteopontin is present. (*B*) Arterial segment free of atheroma.

mally associated with bone formation provides compelling evidence that the nature of calcification of coronary arteries has similar features to the processes that regulate bone formation. Another study has incriminated bone morphogenic protein 2a in association with atherosclerosis; however, its association with the mineralization front was less clear (15).

Preliminary studies have demonstrated that in vitro, cultures of smooth muscle cells derived from porcine coronary arteries expressed type I collagen and the noncollagenous matrix proteins, osteocalcin, osteonectin and osteopontin (36). Several studies have described the presence of mRNA for osteopontin in association with smooth muscle cells of atherosclerotic plaque (37–39). Ikeda et al. (40) has reported the presence of osteopontin mRNA in smooth muscle-derived foam cells and Hirota and colleagues implicate the production of osteopontin mRNA by macrophages in human aortic atherosclerotic lesions (41). Others have found osteopontin in pericyte-like cells cultured from the intima and media of bovine and human aorta (15).

After injury of the vessel, the production of osteopontin by smooth muscle cells of the tunica media (36) may prevent deposition of calcium. The onset and progression of coronary arterial calcification may occur as a result of injury-related stimuli that alter the normal cellular and protein composition of the vascular wall (35, 38, 39, 41, 42). Doherty and Detrano (6) have suggested that coronary artery calcification strengthens the

weakened atherosclerotic plaque. Other investigators suggest that the risk of plaque calcification may not be clinically assessed to date (43). The possibility also exists that in healthy vessels the presence of certain calcium binding proteins reduces the likelihood of calcium deposition. Damage to the vascular endothelium may initiate a cascade of events that results in the expression of cytokines which stimulate cellular proliferation, migration and production of extracellular matrix. Alterations in the extracellular matrix, in turn, may result in the production of procoagulant factors and accelerate the atherosclerotic process (44). Dynamic changes in the matrix composition of atherosclerotic vessles may indirectly make this tissue more susceptible to calcification, which protects the vessel from rupture resulting in certain death.

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