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# MATRIX PROTEINS ASSOCIATED WITH BONE CALCIFICATION ARE PRESENT IN HUMAN VASCULAR SMOOTH MUSCLE CELLS GROWN *IN VITRO*

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## SUMMARY

Atherosclerotic lesions are composed of cellular elements that have migrated from the vessel lumen and wall to form the cellular component of the developing plaque. The cellular elements are influenced by various growth-regulatory molecules, cytokines, chemoattractants, and vasoregulatory molecules that regulate the synthesis of the extracellular matrix composing the plaque. Because vascular smooth muscle cells (VSMC) constitute the major cellular elements of the atherosclerotic plaque and are thought to be responsible for the extracellular matrix that becomes calcified in mature plaques, immunostaining for collagenous and noncollagenous proteins typically associated with bone matrix was conducted on VSMC grown *in vitro*. VSMC obtained from human aorta were grown in chambers on glass slides and immunostained for procollagen type I, bone sialoprotein, osteonectin, osteocalcin, osteopontin, decorin, and biglycan. VSMC demonstrated an intense staining for procollagen type I, and a moderately intense staining for the noncollagenous proteins, bone sialoprotein and osteonectin, two proteins closely associated with bone mineralization. Minimal immunostaining was noted for osteocalcin, osteopontin, decorin, and biglycan. The presence in VSMC of collagenous and noncollagenous proteins associated with bone mineralization suggest that the smooth muscle cells in the developing atherosclerotic plaque play an important role in the deposition of the extracellular matrix involved in calcification of developing lesions.

*Key words:* atherosclerosis; calcification; extracellular matrix proteins; collagen; noncollagenous proteins.

## INTRODUCTION

According to the response-to-injury hypothesis for the development of atherosclerotic lesions, endothelial cell injury leads to migration of mononuclear cells from the vascular lumen into the subendothelial tissue (32). Secretion of various growth-regulatory molecules, cytokines, chemoattractants, and vasoregulatory molecules by endothelial cells and mononuclear cells induces quiescent vascular smooth muscle cells (VSMC) to change their cellular and molecular structure enabling them to migrate into the subendothelial tissue where they enter the cell cycle. When the VSMC are established in the intimal layer of the vessel wall, these cells, along with macrophages, accumulate lipid droplets and form the foam cells. Other VSMC secrete extracellular matrix. The developing atherosclerotic plaque is thus composed of foam cells, scattered extracellular lipid particles, VSMC, and extracellular matrix, with VSMC being the major constituent cell type (34).

It has been proposed that exposure of VSMC to different matrix components may play a role in the vascular wall remodeling that occurs in response to specific changes in the extracellular environment (18). Liptay and colleagues evaluated the role of local extracellular matrix gene expression in atherosclerotic lesions and suggested a role for macrophages in the production of fibronectin and type I procollagen (27). Recently, Shanahan and colleagues demonstrated the presence of two bone-associated proteins, osteopontin and matrix Gla protein, in human atherosclerotic plaques (33). Kaufmann and colleagues demonstrated that extracellular matrices derived from neonatal rat aortic smooth muscle cells altered human monocyte morphology and metabolism (25). Although purified alpha-elastin substratum was responsible for some of the changes, other matrix components were responsible for the changes in monocyte-derived phagocytic cells. Matrices from endothelial cells can alter VSMC growth, contractile phenotype, and sensitivity to heparin (20), and other data suggest that macrophages modulate the VSMC phenotype and matrix (9).

With time, the extracellular matrix produced by the VSMC of some developing atheromatous plaques becomes calcified. Although the mechanism of calcification in arterial plaques is poorly understood, accumulating evidence suggests that pathologic calcification of atherosclerotic vessels shares features with normal bone formation such as cellular proliferation, matrix production, and mineralization. Bone matrix and atherosclerotic plaques contain type I collagen (8,31), and mineral deposits in arterial plaques consist of crystalline hydroxyapatite, the major inorganic component of bone (10). In addition, elaboration by VSMC of noncollagenous proteins typically as-

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sociated with mineralized matrix may be an important constituent of the molecular scaffold responsible for the calcification of atherosclerotic lesions.

In culture, VSMC are phenotypically and physiologically distinct from the differentiated, contractile smooth muscle cells of the normal vascular wall. The cells in culture have properties similar to the smooth muscle cells that have migrated into the developing atherosclerotic plaque where they modulate from the contractile state in the healthy vessel to the synthetic state typical of the atherosclerotic vessel (1,2,3,29). Recent studies have demonstrated a population of cells isolated from human aorta and grown in culture that exhibit high levels of alkaline phosphatase and collagen type I, express osteopontin and osteonectin mRNA, and show intense calcification as demonstrated with von Kossa staining in response to 25-hydroxycholesterol and transforming growth factor-β1 (39). To understand further the calcification process in human atherosclerosis, human vascular smooth muscle cells grown in culture were examined for the presence of extracellular bone matrix proteins using immunostaining techniques.

## Materials and Methods

Aortic VSMC (Clonetics Corp., San Diego, CA) from the sixth to eighth passage were plated on eight-chambered glass slides (Nunc, Inc., Naperville, IL) and grown to near confluence in smooth muscle cell growth medium (SmGM™, Clonetics) containing 5% fetal bovine serum (FBS) plus growth factors. Following thorough washing with phosphate-buffered saline (PBS), the VSMC were cultured for 24 h in serum-free Dulbecco's modified Eagles medium (DMEM), and then cultured for 48 h in DMEM containing 10% FBS with no added growth factors. The cells were rinsed twice in cold PBS and fixed for 10 min in absolute ethanol at −20° C. Slides were air dried and stored at −20° C.

*Antibodies.* Bovine bone-derived osteonectin was used to generate antisera against bone osteonectin. Bovine osteocalcin protein, a generous gift of Dr. Paul Price, was purified by high-performance liquid chromatography prior to use as an immunogen to generate osteocalcin. These antibodies cross-react to their respective human bone-derived proteins (6). Polyclonal anti-biglycan and anti-decorin antibodies were generated against human biglycan and decorin sequence (15). Rabbit polyclonal antiserum for detection of osteopontin and bone sialoprotein was generated against purified human osteopontin and bone sialoprotein, respectively (antibodies were generously provided by Dr. L. Fisher) (14). Procollagen type-I propeptide antibody was obtained from Pharmos Diognistica, Turku, Finland.

*Immunocytochemical staining.* Frozen cultured VSMC attached to the glass slides were rehydrated in 50% ethanol followed by rinsing in Tris-buffered saline [TBS, 0.05% Tris, 0.01% bovine serum albumin (BSA), and 0.9% NaCl, pH 7.5]. The cells were blocked with 10% normal goal serum in TBS for 30 min. Cells were stained using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations with modifications. All steps were carried out at room temperature and were followed by a 30-min wash. Cells were incubated with primary antibody (1:200 in TBS containing 1.5% normal goat serum) for 1 h and washed in TBS containing 0.02% Triton X-100 (TTBS). Primary antibody was detected by incubation with biotinylated secondary antibody (goat anti-rabbit) for 30 min and washed with TTBS. Endogenous peroxidase activity was inhibited with 1.5% $H_2O_2$ and 0.1% sodium azide in 50% methanol for 15 min. Peroxidase-conjugated avidin-biotin complex (ABC) in TBS containing 0.5% NaCl was allowed to react with secondary antibody for 30 min. Antibody complexes were visualized after the addition of 0.01% diaminobenzidine and 0.01% $H_2O_2$ in Tris, pH 7.2, for 4 min. The cells were rinsed with water, dehydrated with ascending alcohols, cleared with xylene, and coverslipped using Eukitt mounting medium (Calibrated Instruments, Hawthorne, NY). Control sections were stained using normal rabbit serum at the same dilution as primary antibody. VSMC stained for the two proteoglycans, biglycan and decorin, were preincubated with chondroitinase ABC (1.25 U/ml in 0.1 *M* Tris and 0.05 *M* calcium sulfate, pH 7.4) for 10 min at 37° C prior to blocking. Chondroitinase ABC enhanced specific staining, as described elsewhere (4).

## Results

Vascular smooth muscle cells immunostained using rabbit immunoglobulin were negative (Fig. 1 *A*). Staining of cells in the early passages confirmed the presence of α-smooth muscle actin (data not shown). Staining for procollagen type I demonstrated an intense cytoplasmic staining in the perinuclear area of the VSMC, but no staining in the peripheral cytoplasmic processes (Fig. 1 *B*). A moderately intense staining for bone sialoprotein, a highly bone-specific protein with a high affinity for binding calcium and hydroxyapatite, was present throughout the VSMC cytoplasm (Fig. 1 *C*). Bone osteonectin, the most abundant noncollagenous protein in bone with a high affinity for binding calcium, hydroxyapatite, and collagen type I, was localized primarily in the perinuclear area of the cytoplasm (Fig. 1 *D*). Osteocalcin and osteopontin, two other proteins localized in bone but ubiquitous in distribution, were minimally stained (Fig. 1 *E,F*). The sulfated proteoglycans, decorin and biglycan that bind TGFβ, were minimally stained, but the cells stained for decorin always demonstrated a moderately intense nuclear staining (Fig. 1 *G,H*). The relative staining for the various proteins are summarized in Table 1.

## Discussion

Inappropriate cellular proliferation and synthesis of matrix proteins is a hallmark of vascular disease. One hypothesis suggests that myocytes undergo phenotypic conversion, perhaps initiated by the exposure of smooth muscle cells to paracrine factors. In turn, the VSMC produce extracellular matrix that can regulate the dedifferentiation and proliferation of additional VSMC. Previous studies have indicated the presence of mRNA capable of encoding for types I, III, IV, V, VI collagens, fibronectin, and laminin in VSMC derived from iliac arteries or the inferior vena cava (28,36). Recent work has implicated the interaction of matrix proteins with lipid components such as LDL and Lp(a) leading to the focal retention and aggregation of atherogenic proteins in the arterial wall (35). Additional evidence supports the importance of the matrix component in the interactions occurring between VSMC and the soluble growth mediators (38).

It is plausible that the properties, synthesis, and deposition, of many of the bone matrix proteins that become mineralized by the precipitation of carbonate-containing hydroxyapatite are applicable to the processes involved in the calcification of atherosclerotic lesions. Mineralized bone matrix contains predominantly type I collagen, although other collagen types have been detected at very low quantities. The role of type I collagen in calcification remains elusive, since type I collagen fibrils may be coated with other collagen types in addition to proteoglycans. The presence of type I collagen and noncollagenous proteins are features common to normal bone calcification (23) and calcification associated with coronary atherosclerosis (7,8,31). Immunostaining of VSMC for type I collagen confirms the synthetic capabilities of the cells in culture.

Immunostaining of VSMC in culture for noncollagenous proteins associated with calcification of bone matrix supports the role of VSMC in initiating calcium deposition in the atherosclerotic lesion. Bone sialoprotein (BSP), first isolated in intact form and characterized by Fisher et al. (16), constitutes about 10% of the noncollagenous bone proteins. It is almost exclusively produced by osteoblasts, osteocytes, and a particular subset of hypertrophic chondrocytes and appears coincidently with the onset of calcification (5). Although the precise role of BSP in calcification is unknown, expression of the protein in bone is essentially restricted to cells directly involved in

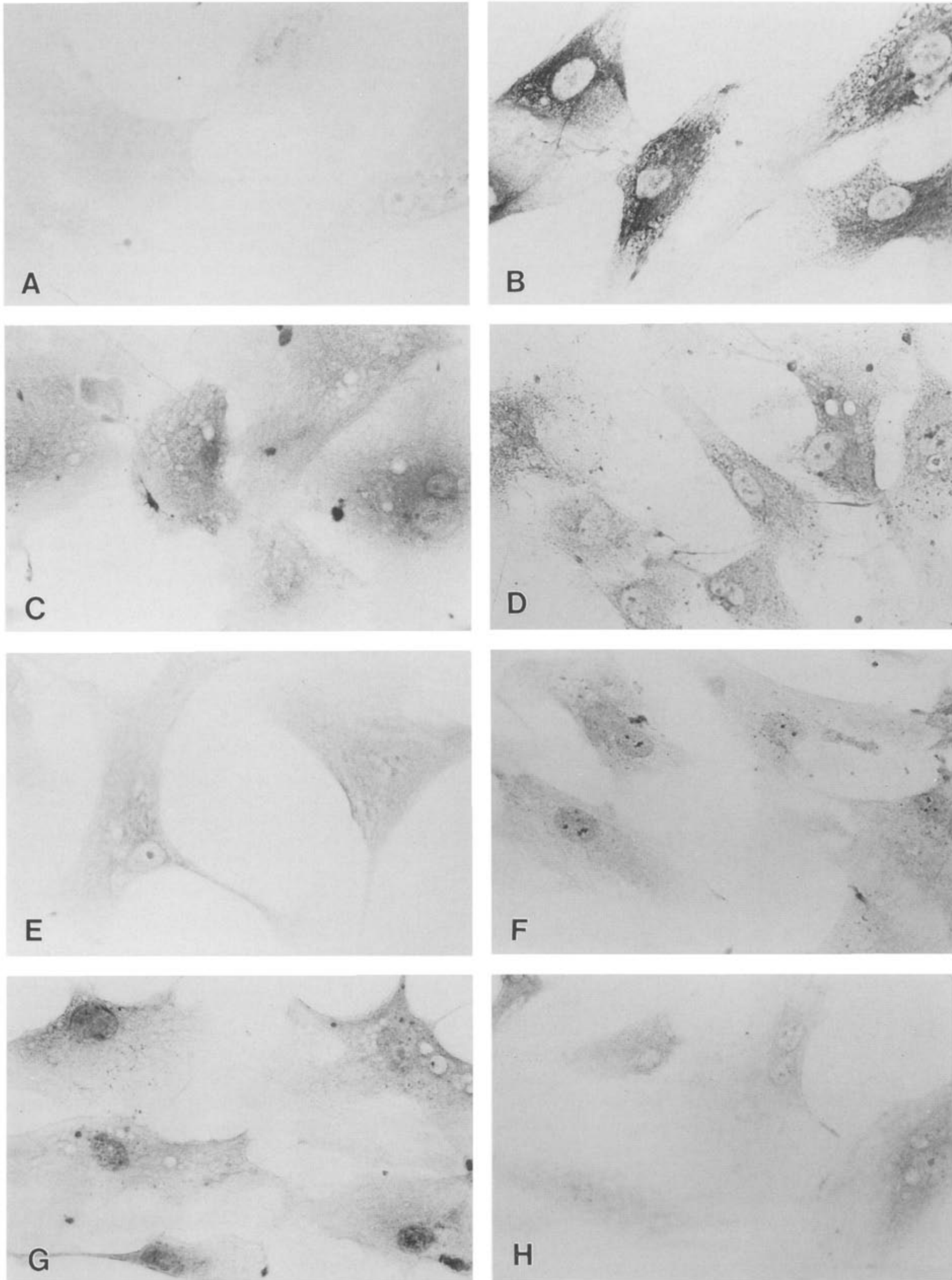


FIG. 1. Vascular smooth muscle cells immunostained for extracellular matrix proteins: *A*, Control; *B*, Procollagen type I; *C*, Sialoprotein; *D*, Osteonectin; *E*, Osteocalcin; *F*, Osteopontin; *G*, Decorin; *H*, Biglycan. ×300.

TABLE 1

RELATIVE IMMUNOSTAINING OF HUMAN VASCULAR SMOOTH MUSCLE CELLS

| Antibody | VSMC |
|---|---|
| Normal Rabbit Serum | − |
| Procollagen type I | + + + |
| Bone Sialoprotein | + + |
| Bone Osteonectin | + + |
| Osteocalcin | + |
| Osteopontin | + |
| Decorin | + |
| Biglycan | + |

the calcification of the extracellular matrix, indicating that BSP could have a specific role in the initiation or process of calcium deposition in both bone and mature atherosclerotic lesions (11,12). Likewise, VSMC stained for osteonectin, the most abundant glycoprotein in bone matrix with a high affinity for calcium, hydroxyapatite, and type I collagen (37). Using *in situ* hybridization, Hirota et al. (21) localized bone osteonectin mRNA to smooth muscle cells of the aortic media in young individuals, but reported its absence in the media of adult aortas with atherosclerosis; however, some smooth muscle cells that had invaded the atherosclerotic lesion expressed bone osteonectin mRNA. The presence of two prominent proteins associated with bone in cultured VSMC supports the role of these cells as contributors to the synthesis of matrix involved in the calcification of atherosclerotic lesions.

Two other bone matrix proteins, osteocalcin and osteopontin, were minimally stained, and probably play a less significant role in the early stages of atherosclerotic lesion development. Osteocalcin is a bone-specific protein produced at the very endstage of bone formation, perhaps even after mineralization has occurred (24). Osteocalcin is synthesized by mature osteoblasts and osteocytes, and regulates the recruitment and activity of osteoclasts, the bone-resorbing cells, and their precursors (26). Osteopontin can be detected in bone using immunocytochemical techniques (19,23) and is found in high concentration at the bone mineralization front (30). Osteopontin mRNA expression was found to be minimal in the aortas of young individuals, but increased with the development of atherosclerotic lesions (21). Macrophages located in the aortic subendothelial tissue of young individuals expressed osteopontin mRNA, while macrophages located at other sites did not express osteopontin mRNA (21). Neither Northern blot analysis or *in situ* hybridization revealed expression of osteopontin mRNA in normal aorta; however, osteopontin mRNA was detected by both methods in sites of atherosclerotic lesions, with a close relationship between calcification and *in situ* localization of osteopontin mRNA in smooth muscle-derived foam cells (22). Recent studies have detected the expression of genes encoding for osteopontin and matrix Gla protein in both smooth muscle cells and macrophages present in human atherosclerotic plaques (33). Matrix Gla protein is a vitamin K-dependent protein with high affinity for hydroxyapatite and is thought to regulate mineralization in bone and cartilage. Interaction of these proteins with lipid may be an important ideologic mechanism in the development of plaque formation. Vascular smooth muscle cells in uninjured arteries contained very low levels of osteopontin protein and mRNA, while injury enhanced expression of osteopontin protein and mRNA in arterial smooth muscle (17). Because immunostaining of cultured VSMC for osteopontin protein was minimal, the VSMC in culture appear similar to the cells of uninjured arteries.

During the process of osteogenesis, a large chondroitin sulfate proteoglycan (CSPG) is replaced by two small CSPGs, biglycan and decorin (13). Biglycan and decorin are the most abundant proteoglycans found in mineralized matrix. Although biglycan and decorin are the product of separate genes, their protein cores are highly homologous and contain multiple repeats of a leucine-rich sequence (15). This sequence is also found in a number of other proteins that are cell surface associated and involved in morphogenesis. These proteins bind to TGF-β and perhaps regulate its activity. Their functional role remains elusive and the significance of these proteins in VSMC is unknown.

Further study of the selective stimulation or inhibition of the expression of these matrix proteins by various autocrine and paracrine growth factors may allow insight into the mechanisms by which these proteins alter proliferation and growth of VSMC. An understanding of extracellular matrix production by VSMC could result in the development of strategies directed at influencing extracellular matrix synthesis and provide innovative therapeutic approaches to many vascular disorders.

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