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Research Article

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Bone Morphogenetic Protein Expression in Human Atherosclerotic Lesions

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Abstract

Artery wall calcification associated with atherosclerosis frequently contains fully formed bone tissue including marrow. The cellular origin is not known. In this study, bone morphogenetic protein-2a, a potent factor for osteoblastic differentiation, was found to be expressed in calcified human atherosclerotic plaque. In addition, cells cultured from the aortic wall formed calcified nodules similar to those found in bone cell cultures and expressed bone morphogenetic protein-2a with prolonged culture. The predominant cells in these nodules had immunocytochemical features characteristic of microvascular pericytes that are capable of osteoblastic differentiation. Pericyte-like cells were also found by immunohistochemistry in the intima of bovine and human aorta. These findings suggest that arterial calcification is a regulated process similar to bone formation, possibly mediated by pericyte-like cells. (*J. Clin. Invest.* 91:1800–1809.) Key words: calcification • pericyte • smooth muscle • intima • artery wall

Introduction

In 1863, Virchow noted that the mineral component in calcified arteries was "an ossification, and not a mere calcification; the plates which pervade the inner wall of the vessel are real plates of bone . . . we see ossification declare itself in precisely the same manner as when an osteophyte forms on the surface of bone . . . following the same course of development (1)." Calcification is a prominent feature of coronary atherosclerosis (2) and correlates with increased risk of myocardial infarction (3), possibly caused by loss of distensibility (4), vasomotion (5), or compensatory enlargement (6). Artery wall calcification takes the form of hydroxyapatite mineral (7, 8), is similar biochemically and ultrastructurally to bone tissue (8), and may include hematopoietic marrow (9). It may promote endothelial injury and plaque rupture by reducing shock absorbance and focusing solid shear stresses on the lesion by introducing an interface between compliant and rigid components (4).

To evaluate whether arterial calcification occurs by a process similar to bone formation, we examined calcified human

plaque by in situ hybridization and found expression of bone morphogenetic protein-2a (BMP-2a)[1], a potent osteogenic differentiation factor (10). We then developed an in vitro model of arterial calcification using cultured human and bovine aortic cells. In culture, calcification colocalized in nodules with cells having morphologic and immunocytochemical features of microvascular pericytes (11) and expressing BMP-2a with prolonged culture. Pericyte-like cells were also found in bovine and human aortic intima by immunohistochemistry.

Methods

In situ hybridization. Human carotid endarterectomy specimens were obtained from three patients with hemodynamically significant lesions. By preoperative two-dimensional ultrasonic examination, all three lesions were complex and moderately to severely calcified. Their stenosis severities measured 70, 80, and 90–95%, respectively. Specimens were obtained within 15 min of removal, then frozen in OCT compound (Tissue-Tek, Elkhart, IN) using liquid nitrogen. Cryosections (7 μm) were thaw mounted on treated glass slides (Superfrost®/Plus, Fisher Scientific, Pittsburgh, PA) and post fixed in 4% paraformaldehyde for ~ 20 min. Nonradioactively labeled riboprobes for bone morphogenetic proteins were prepared by in vitro transcription of pBluescript II SK M13 (+) (Stratagene, La Jolla) containing the partial cDNA (12) (kindly provided by Dr. E. Wang, Genetics Institute, Cambridge, MA) for either human BMP-2a (nucleotides 1205–1547) or BMP-3 (nucleotides 1208–1774) inserted into the PstI and XbaI sites of the plasmid. In vitro transcription was performed in the presence of digoxygenin-11-dUTP, according to the manufacturer's instructions (Genius®; Boehringer-Mannheim Biochemicals, Indianapolis, IN). For antisense riboprobes, the plasmids were linearized with EcoRI and transcribed with T3 RNA polymerase; for sense riboprobes, they were linearized with SstI and transcribed with T7 RNA polymerase. Sections of endarterectomy specimens were treated with proteinase K, prehybridized and hybridized with the labeled riboprobes. Unhybridized probe was removed by incubation with RNase A. Specimens were washed under stringent conditions. After hybridization, specimens underwent four 10-min washes in 2× SSC, 30 min immersion in RNase A (20 μg/ml), eight washes of 0.1× SSC at 55°C (for a total of 4 liters) over 2 h, and two additional 10-min washes in 0.5× SSC. Probe binding was localized by a colorimetric reaction with an alkaline phosphatase–conjugated antidigoxygenin antibody (Genius®, Boehringer-Mannheim). Endogenous alkaline phosphatase was blocked using levamisole (0.1 mM). For negative controls, serial sections were processed (*a*) without probe; (*b*) with the corresponding sense riboprobe; and (*c*) after RNase A treatment.

In situ hybridization of aortic medial cell cultures with and without nodules were performed using cultures in gelatin-coated two-well chamber slides (Nunc, Naperville, IL). For in situ hybridization of sectioned nodules, well-formed nodules were separated from the cultures using a scalpel. They were fixed in 4% paraformaldehyde for a
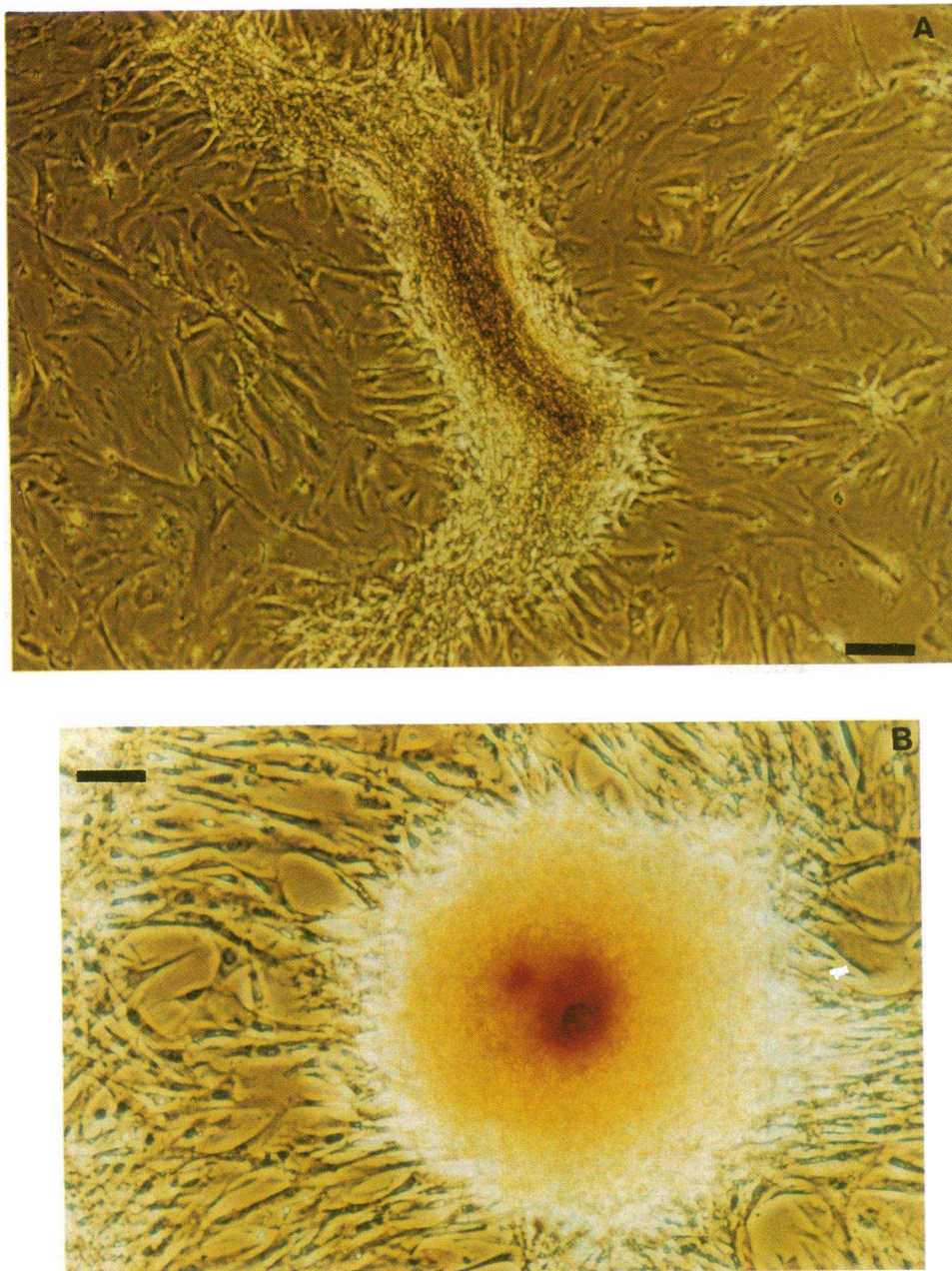
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1. *Abbreviation used in this paper:* BMP, bone morphogenetic protein.
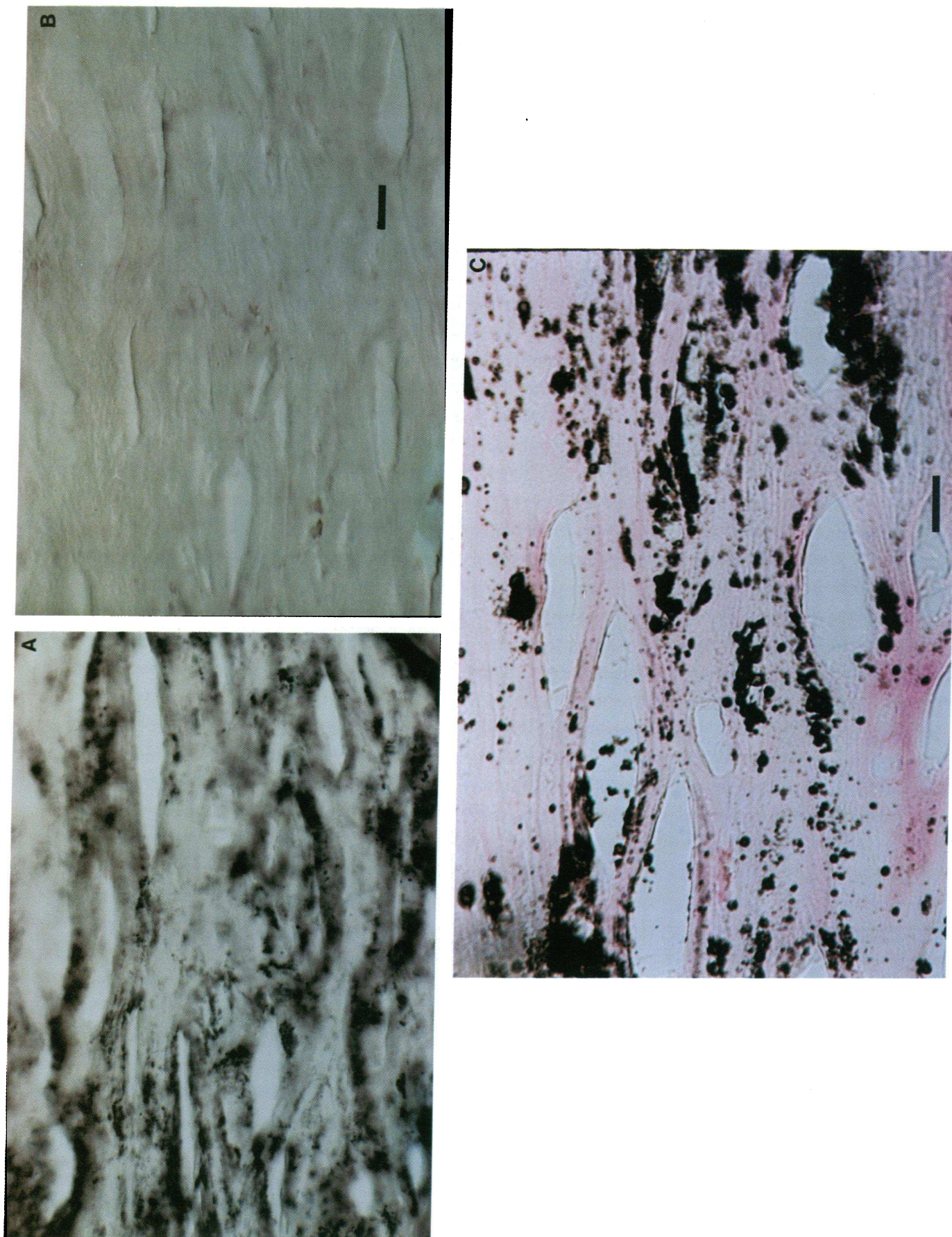
Figure 1. In situ hybridization using digoxygenin-labeled antisense (*A*) and sense (*B*) riboprobes for human BMP-2a in calcified human carotid atherosclerotic lesion. (*C*) Corresponding von Kossa-stained section in which calcification stains black. Bar, 25 μm.

Figure 2. Nodules formed by cultured aortic medial cells. (*A*) Advanced human aortic medial cell nodule at 6 wk (phase contrast). (*B*) Bovine medial cell nodule with calcification (red) at 5 wk (Alizarin red S stain, phase contrast; bar, 50 μm).

minimum of 12 h and embedded in paraffin. Horizontal, 5-μm sections were placed onto treated glass slides (Superfrost®/Plus, Fisher) using RNAse precautions. After dewaxing with xylene and rehydration, the remainder of the in situ hybridization protocol was the same as for the endarterectomy specimens.

Tissue culture. Bovine and human thoracic aortic medial cells were cultured from explants sectioned from the luminal face of the aortic media (13). Small sections were obtained from bovine thoracic aorta or human cardiac transplant donor aorta. Tissue was rinsed in serum-free medium, dissected free of adventitia, and placed in 0.2% type I collagenase in a 1:1 mix of medium and PBS for 30 min at 37°C. Endothelium was gently removed using a plastic cell scraper. The medial layer was isolated in ice-cold PBS, and 1-mm^2 explants were obtained from the luminal portion. The explants were placed in gelatinized tissue culture dishes and allowed to adhere for 15–30 min then covered with a coverslip. Growth media consisted of M199 medium for human cells, or DME for bovine cells, supplemented with 15% heat-in-

activated FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.25 μg/ml fungizone, and 25 mM Hepes buffer, adjusted to pH 7.25. After 5–7 d, the medial cells had grown onto the plastic, the explant was removed, and the cells were allowed to grow. Additional passages of bovine aortic medial cells were grown in McCoy's medium supplemented with 15% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml fungizone without affecting results.

To characterize the cellular composition of nodules formed in smooth muscle cell cultures, the nodules were disrupted into individual cells by enzymatic treatment. First, cultures containing nodules were detached using trypsin (0.1% in 2 mM EDTA) leaving the nodules intact. This suspension was diluted in serum-free medium and centrifuged for 30–45 s at 150 g. The pellet of nodules was dispersed using collagenase (0.2% type I for 30 min), seeded at 10,000 cells/cm^2 on gelatin-coated chamber slides (Nunc) and grown to near confluency. To obtain clones from individual cells, cultures of nodule-derived cells
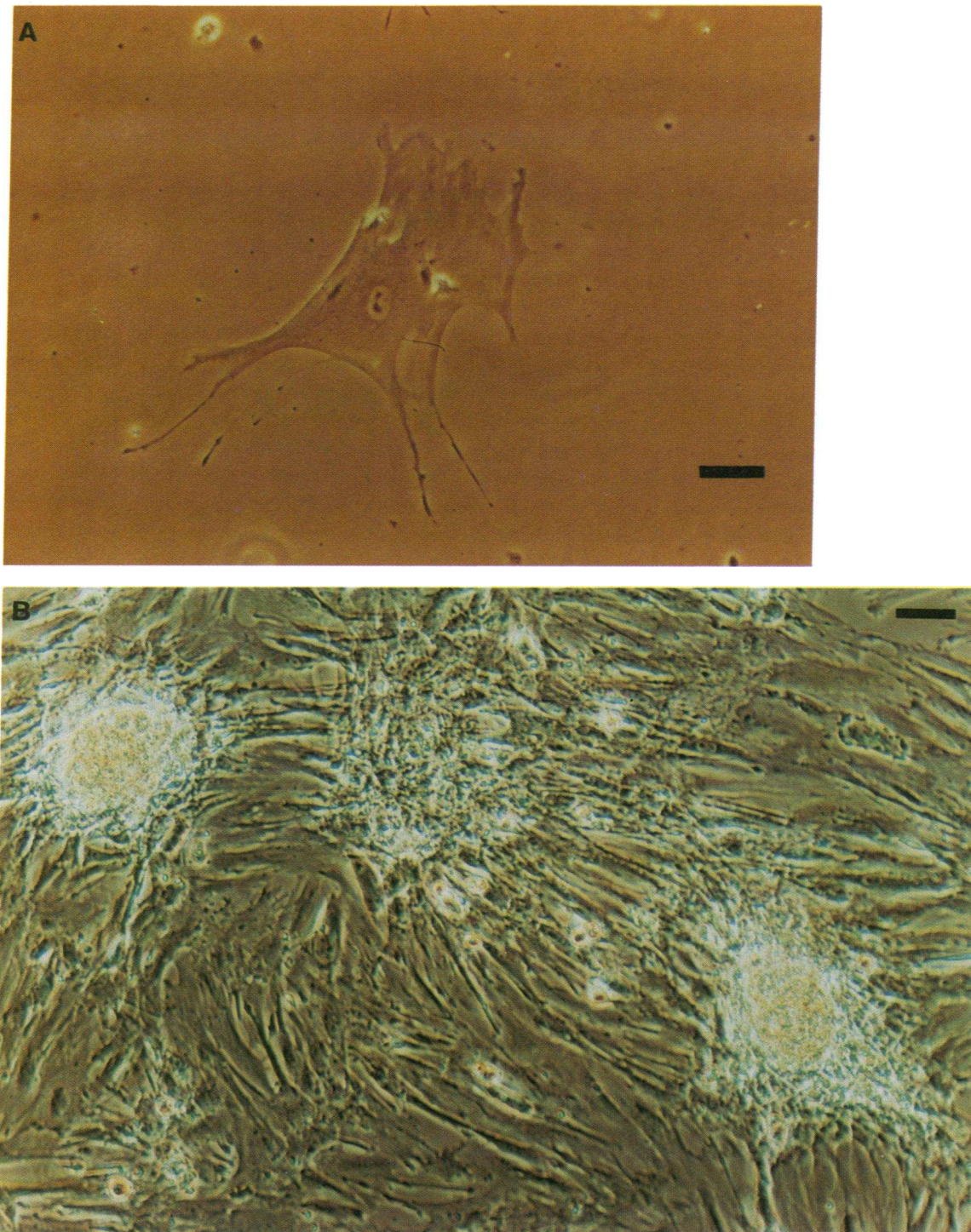
Figure 3. Cells derived from a nodule formed by bovine aortic medial cells. (*A*) Individual cell from a nodule showing pericyte-like morphology (phase contrast; bar, 3 μm). (*B*) High density nodules forming after 3–5 d in nodule-derived cells (phase contrast; bar, 50 μm).

were resuspended in 0.1% trypsin in 2 mM EDTA in PBS, washed, and cloned (14). Individual clones were detached using trypsin and cloning rings or gentle scraping, replated and cultured in conditioned medium for several days, then DME or McCoy's medium for 1 to 4 mo. Cells were used in experiments between passages 8 and 21.

Trypsin and PBS were purchased from Gibco (Grand Island, NY); gelatin from Sigma Immunochemicals (St. Louis, MO); collagenase from Worthington Biochemical (Freehold, NJ); M199 media from MA Bioproducts (Walkersville, MD); DME and McCoy's medium from Irvine Scientific (Irvine, CA); Triton X-100 from Mallinckrodt (Paris, KT); FBS from Hyclone (Logan, UT); and tissue culture plates from Costar Corp. (Cambridge, MA).

Electron microscopic analysis. For transmission electron micros- copy, cultured bovine aortic medial cells with nodules were fixed di-
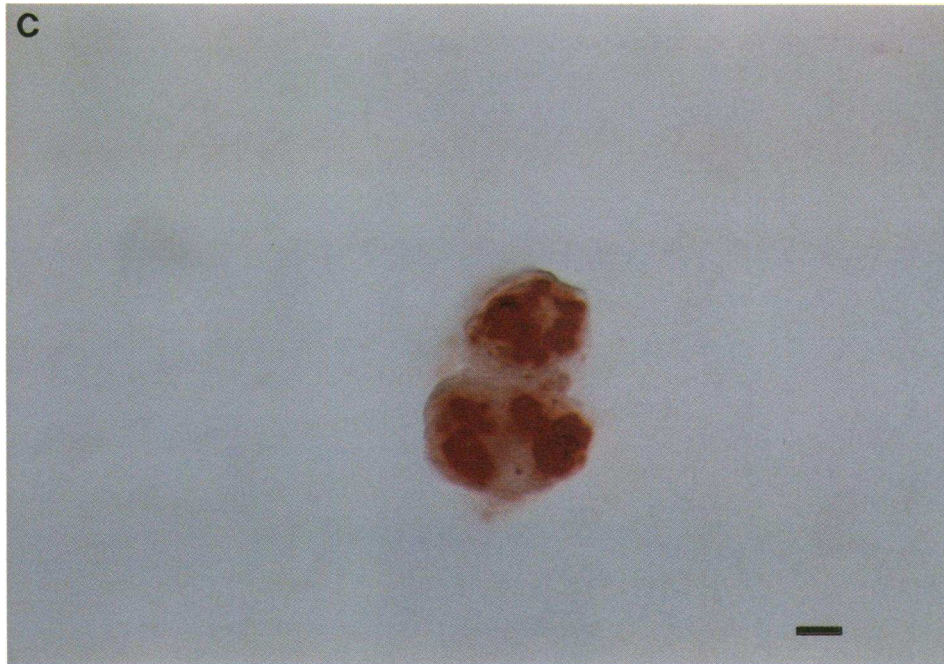
Figure 3 (*Continued*). (*C*) Calcified nodule formed by clone of pericyte-like, nodule-derived cell (Alizarin red S stain, light microscopy; bar, 40 μm).

rectly on the culture dishes with 2% glutaraldehyde in 0.2 M cacodylate for 1 h, postfixed with 1% osmium tetroxide in PBS for 30 min, dehydrated with ethanol, and embedded in Epon. Thin sections (1,000 Å) were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (JEOL-100 CX; JEOL, Ltd., Tokyo).

For electron microprobe analysis, the mineralized nodules were processed and sectioned (1,000 Å) in the same manner as for transmission electron microscopy except that they were carbon coated before examination for stability, and they were not osmicated or stained. They were probed using a JEOL-100 CX microscope equipped for x-ray microanalysis, in the scanning transmission mode at 100 kV acceleration voltage. The probe was rastered for 100 to 200 s over 12 different electron-dense regions of the sections at a range of magnifications. The x rays generated were analyzed by an energy dispersive spectrometer (Fully Quantitative Link AN 10000; Oxford Instruments, Oak Ridge, TN).

Immunocytochemistry. For immunocytochemistry using mouse antihuman smooth muscle alpha actin (Dako Corp., Carpinteria, CA) and rabbit anti-nonmuscle actin (15), the cells were fixed in acetone/methanol (1:1) at −20°C for 20 min, 0.1% Triton X-100 (Sigma) for 5 min, and primary antibody (1:300) with 0.05% Tween-20 at 25°C for 90–120 min. Mouse mAb 3G5 to pericyte surface ganglioside (16) (gift of R. Nayak, Harvard Medical School, Boston, MA) was applied (1:40) to unfixed cells. After washing, FITC-conjugated secondary antibody (rabbit anti–mouse [Dako] or goat anti-rabbit [Sigma]) was added at 1:30 for 1 h. After rinsing, mounting medium and a cover slip were added, and the cells examined by fluorescence microscopy (Axioscope 20; Carl Zeiss, Inc., Thornwood, NY).

For immunohistochemistry, 5-μm frozen sections of human donor and bovine aorta were prepared, thaw mounted on treated glass slides (Superfrost®/Plus, Fisher Scientific), and were postfixed in 4% formaldehyde (Sigma). Primary antibodies were used at the following dilutions: anti–von Willebrand factor 1:100, anti-alpha smooth muscle actin 1:2,000, and mAb 3G5 at 1:300. All dilutions were made in PBS containing 2% BSA (Sigma). To identify endothelial cells, anti–von Willebrand factor antibodies were applied overnight, secondary antibodies were applied for 30 min, the rabbit peroxidase antiperoxidase

complex (Dako) was applied at 1:100 for 30 min, and the reaction was developed using 3-amino-9-ethylcarbazole (Sigma). For immunostaining with anti-alpha smooth muscle actin and mAb 3G5, the avidin-biotin-peroxidase complex method was used. Primary antibody was applied overnight, biotinylated secondary antibodies were applied for 30 min, then an avidin-biotinyl-peroxidase complex (HRP-Streptavidin; Zymed Laboratories; San Francisco, CA) was applied for 30 min. The reaction was developed as in the peroxidase antiperoxidase method. For both methods, washes were performed between all antibody steps using Tris-buffered saline (pH 7.4). Hematoxylin was used for counterstaining.

Results

Human calcified atherosclerotic lesions contained message for bone morphogenetic protein BMP-2a in areas of calcification (Fig. 1), but not BMP-3 (not shown). Calcification was primarily located at the base of the plaque, but was also present as small foci of calcification in other scattered locations in each specimen. Results were not changed when the length of RNA probes was adjusted to a mass average of ∼ 100–150 bases by partial alkaline hydrolysis (17).

After 10–14 d, cultures of human and bovine aortic smooth muscle cells formed distinct nodules (Fig. 2) similar to those formed by bone and other mesenchymal cells (18). The cells increased in density, overgrew, contracted, drew in neighboring cells, generated matrix material and formed smooth-surfaced nodules at a local density of ∼ 5–10/cm². After 12–16 d, nodules developed multifocal calcifications, occupying up to 30% of nodule diameter, demonstrated by Alizarin red S (Fig. 2 B) and von Kossa histochemical staining, which are generally considered fairly specific demonstrators of calcium mineral (19, 20, 21, 22).

By transmission electron microscopy, nodules consisted of microfibrillar matrix material and cells with abundant assorted
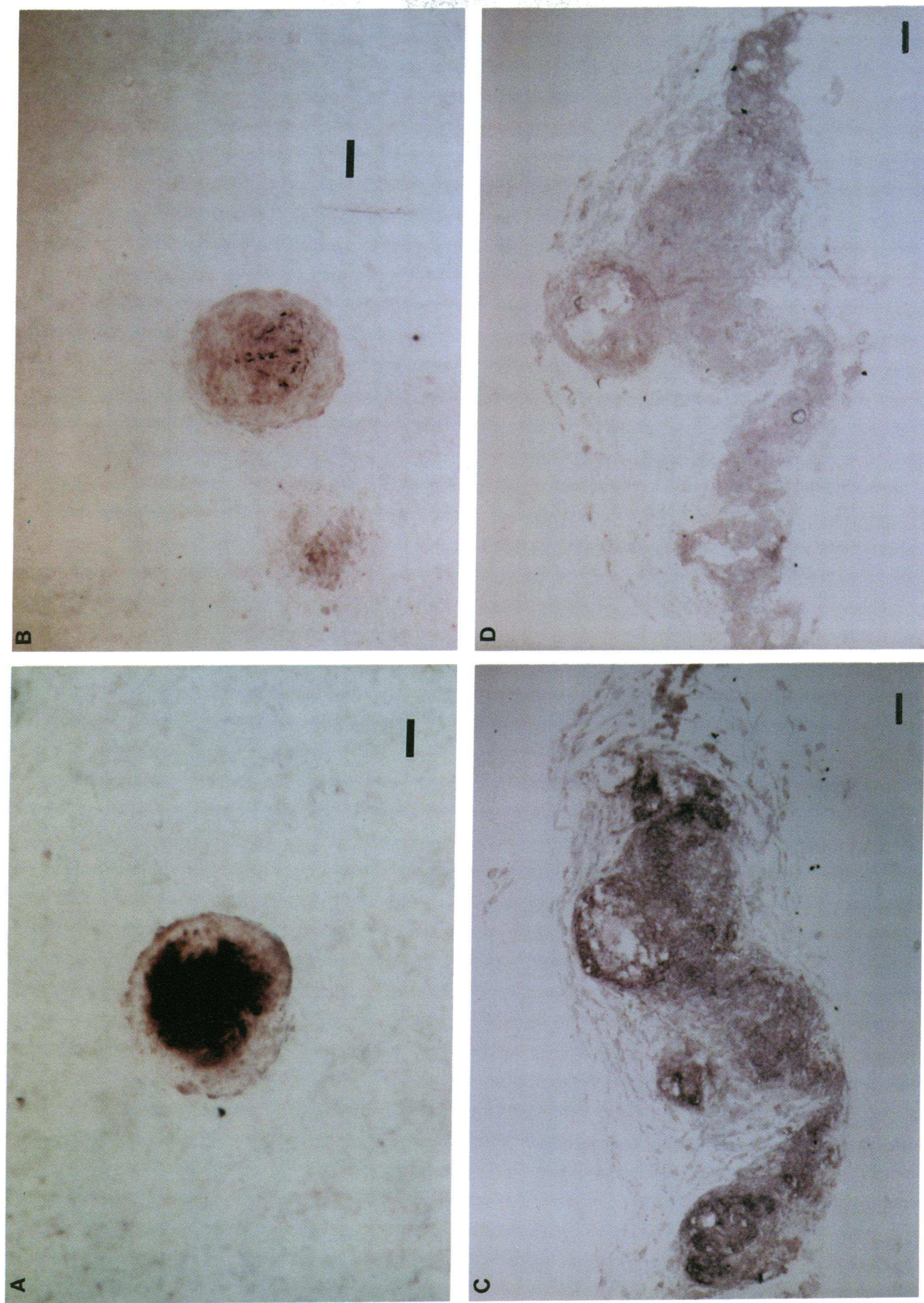
Figure 4. (*A*) In situ hybridization of nodule formed by aortic medial cells grown for > 65 d. Bar, 25 μm. Nodules grown for shorter periods were negative. (*B*) Corresponding sense control, same magnification. (*C*) In situ hybridization of sectioned nodule formed by cloned cell cultures with pericyte-like morphology. Bar, 40 μm. (*D*) Corresponding sense control, same magnification.
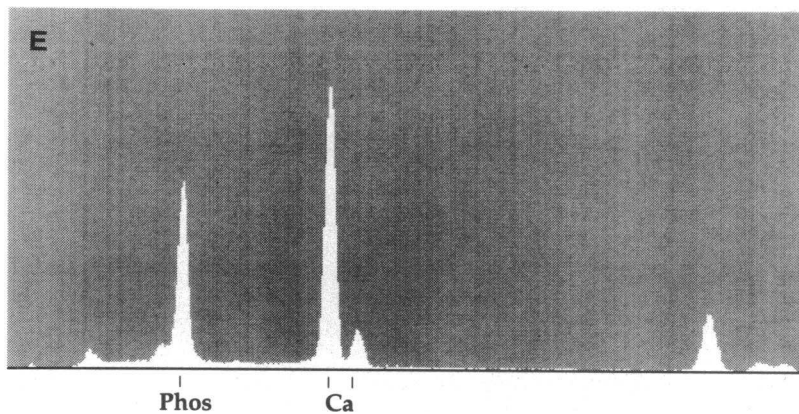
Figure 4 (Continued). (*E*) Spectrum from electron microprobe analysis of electron-dense regions of a nodule formed by bovine aortic medial cells grown for 65 d (1,000 Å section). The content is primarily calcium and phosphate in a ratio of 1.2:1.6, which is consistent with presence of hydroxyapatite. *Phos,* phosphorus peak; *Ca,* calcium peak.

vesicles containing similar microfibrils. The nodule surface was lined with fusiform, lipid-laden cells resembling smooth muscle cells.

Nodules dispersed by collagenase were composed almost entirely of large, flat stellate cells with long, slender side processes, membrane ruffling and short, broad filopods (Fig. 3 *A*). The clones derived from these nodules also formed nodules and shared these morphologic features. Cultures of these cells had a lag phase of ~ 6 d before logarithmic growth and formed new nodules more rapidly (within 3–5 d) and at a several-fold greater density of nodules than did primary cultures (Fig. 3 *B*). These cloned cell culture nodules also developed calcification (Fig. 3 *C*).

In situ hybridization of smooth muscle cell cultures in the absence of nodule formation were negative for BMP-2a and BMP-3. Early nodules formed by smooth muscle cell cultures and by cloned cell cultures in < 45 d were also negative for both. Intact and sectioned nodules from two clones allowed to grow > 65 days were positive for BMP-2a (Fig. 4 *A–D*).

Electron microprobe analysis of sections of calcified nodules demonstrated the mineralized components to be composed primarily of calcium and phosphorus (Fig. 4 *E*) in a molar ratio of 1.19–1.62 (1.30±0.12 [SD]).

Immunofluorescence staining patterns of the pericyte-like cells cloned from aortic medial cell nodules were positive for alpha actin, beta actin, and the epitope of mAb 3G5 in filamentous, granular, and peripheral patterns, respectively (Fig. 5). These features differed from those of cultured human and bovine aortic endothelial cells and smooth muscle cells and human fibroblasts, but were identical to those of microvascular pericytes (Table I).

By immunohistochemistry, frozen sections of human donor and bovine aorta contained mAb 3G5-positive cells in scattered locations within the intima (Fig. 6). In serial sections, the endothelium stained positively for von Willebrand factor, and the media stained positively for alpha actin, as expected. The intima also stained positively for alpha actin, but with approximately half the staining intensity of the media. All three layers stained positively for beta actin. The adventitia also stained positively for mAb 3G5.

Discussion

To our knowledge, this is the first report of bone morphogenetic protein expression in human artery wall or in any soft tissue calcification, and the first demonstration of in vitro calcification of artery wall cells. The mineral content is consistent with hydroxyapatite, which, in its pure form, has a molar ratio of calcium to phosphate of 1.6. The slightly lower ratio observed may be caused by additional phosphorus in the form of phospholipids which are known to be associated with sites of mineralization. The cell producing BMP-2a and calcium mineral is not known. However, in the present study, the calcified nodules formed by artery wall cells resemble those formed by microvascular pericytes (23) and contain cells resembling pericytes in morphology, growth characteristics, and presence and pattern of staining for actin isoforms (15) and surface ganglioside (16). Pericyte-like cells have not previously been demonstrated in the luminal media of a large artery. They may be related to myointimal cells, the "pi" cell described by Schwartz and colleagues (24), mesenchymal precursor cells, or pericytes themselves (25), which are capable of osteoblastic differentiation (26, 27). It is unlikely that they are contaminant pericytes derived from the vasa vasora of the adventitia or outer media, because the explants were derived from the luminal media of presumably normal human and bovine aorta. Their abundance at sites of in vitro calcification suggests a role in calcification. These findings support the concept that arterial calcification is an organized process with similarities to bone formation, rather than a passive precipitation of calcium phosphate

Table I. Immunofluorescence Staining of Cultured Vascular Cells

	Anti–von Willebrand factor	Anti–alpha-actin	Anti–beta-actin	mAb 3G5
Cloned cells from BASMC nodules	−	+	+	+
Pericytes from bovine retina	−	+	+	+
Endothelial cells from bovine/human aorta	+	−	+	−
Smooth muscle cells from bovine/human aorta	−	+	±	−
Fibroblasts, human	−	−	+	−
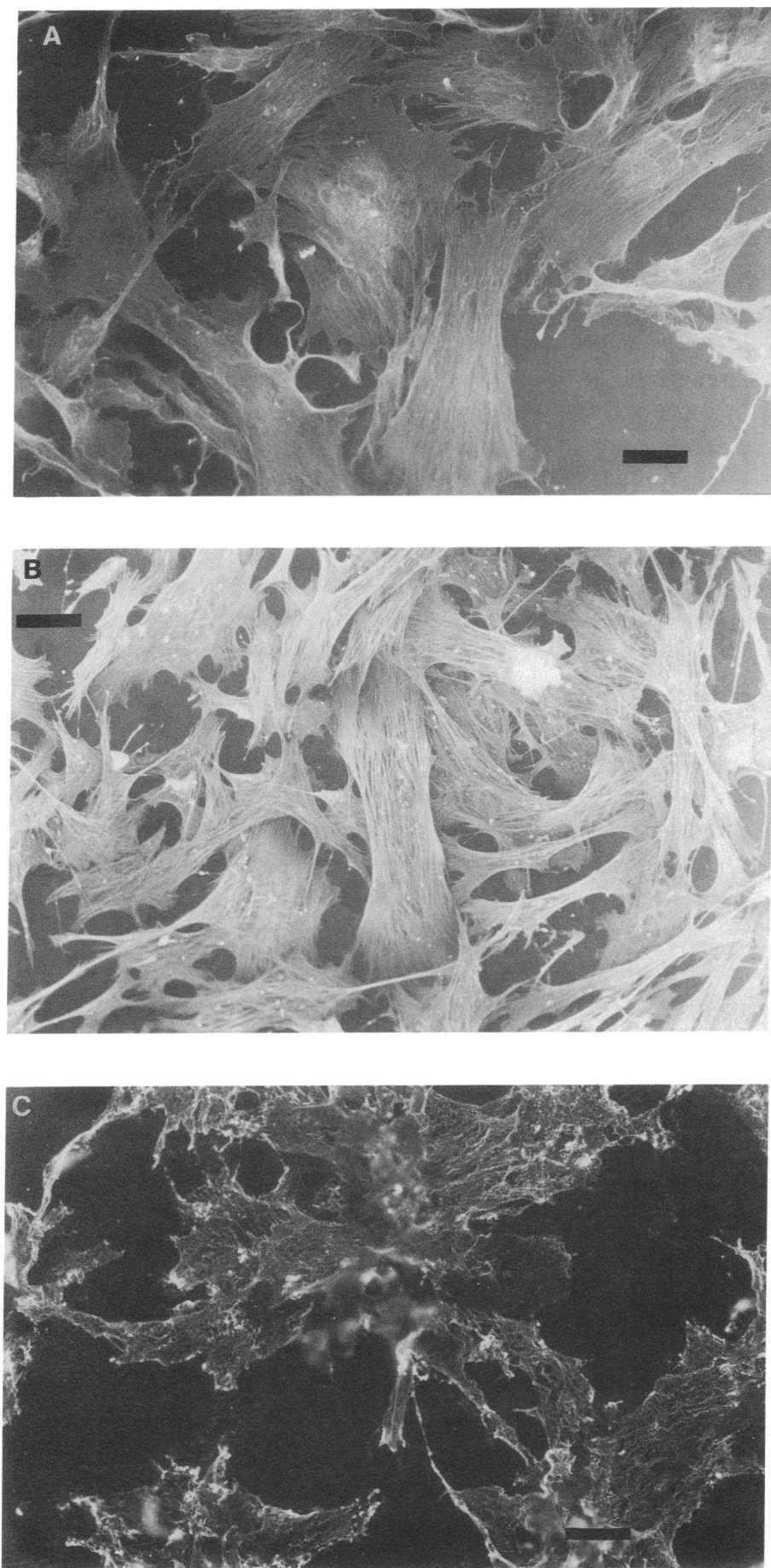
Figure 5. Immunofluorescent staining of cloned cells derived from aortic smooth muscle cell nodules with (*A*) rabbit anti-nonmuscle actin, (*B*) mouse anti–smooth muscle alpha-actin, and (*C*) mouse monoclonal antibody 3G5. Bar, 12 μm. Positive staining for these three markers is characteristic of pericytes. Smooth muscle cells and fibroblasts stained negatively with mAb 3G5 (not shown).

Figure 6. Human thoracic aorta (frozen section) stained by immuno-histochemistry with labeled mAb 3G5 to identify pericyte-like cells. Occasional positive cells were present within the region of mild intimal hyperplasia. Similar distribution was found in bovine aortic sections.

crystals, opening the possibility of preventive or therapeutic interventions.

Acknowledgments

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