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### Research Article

Previous studies in our laboratory demonstrated messenger RNA for bone morphogenetic protein-2a in human calcified plaque, suggesting that arterial calcification is a regulated process, similar to osteogenesis. To further test this hypothesis, we have isolated and cloned a subpopulation of cells from bovine aortic media that show osteoblastic potential. These novel cells are primarily distinguished from smooth muscle cells by expression of a surface marker preliminarily identified as a modified form of the ganglioside sialyl-lactosylceramide (GM3). Osteoblastic potential was indicated by high levels of alkaline phosphatase and collagen I, expression of osteopontin and osteonectin (SPARC), and production of bone-specific osteocalcin and hydroxyapatite. Cultures of these cells were stimulated to form increased numbers of calcium-mineral-producing nodules by the oxysterol 25-hydroxycholesterol as well as by transforming growth factor-beta 1, both known to be present in atherosclerotic lesions. The stimulation of calcifying vascular cells in the artery wall by these two factors suggests a possible mechanism for the colocalization of calcification with atherosclerosis *in vivo*.

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# TGF- $\beta$ 1 and 25-Hydroxycholesterol Stimulate Osteoblast-like Vascular Cells to Calcify

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## Abstract

Previous studies in our laboratory demonstrated messenger RNA for bone morphogenetic protein-2a in human calcified plaque, suggesting that arterial calcification is a regulated process, similar to osteogenesis. To further test this hypothesis, we have isolated and cloned a subpopulation of cells from bovine aortic media that show osteoblastic potential. These novel cells are primarily distinguished from smooth muscle cells by expression of a surface marker preliminarily identified as a modified form of the ganglioside sialyl-lactosylceramide (GM3). Osteoblastic potential was indicated by high levels of alkaline phosphatase and collagen I, expression of osteopontin and osteonectin (SPARC), and production of bone-specific osteocalcin and hydroxyapatite. Cultures of these cells were stimulated to form increased numbers of calcium-mineral-producing nodules by the oxysterol 25-hydroxycholesterol as well as by transforming growth factor- $\beta$ 1, both known to be present in atherosclerotic lesions. The stimulation of calcifying vascular cells in the artery wall by these two factors suggests a possible mechanism for the colocalization of calcification with atherosclerosis in vivo. (*J. Clin. Invest.* 1994; 93:2106-2113.) Key words: atherosclerosis • calcification • oxysterol • pericytes • gangliosides

## Introduction

Calcification is a prominent feature of atherosclerosis (1), frequently associated with myocardial infarction and other adverse cardiovascular outcomes (2-6). Currently, calcification is widely viewed as an end-stage, degenerative process which is inevitable in advanced atherosclerosis. However pathologists have long noted that calcification may occur early in atherosclerosis and, at times, may appear histologically similar to organized bone, including areas resembling bone marrow (7). In addition, matrix vesicles, the nucleation sites for the beginning of organized bone formation have been detected within atherosclerotic lesions (8). These observations suggest that rather than being a passive process, atherosclerotic calcification may instead be an active, regulated process similar to that of osteogenesis.

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Though significant morphologic data exist regarding atherosclerotic calcification, little is known about the molecular or cellular mechanisms involved. In the present study, we have identified cells present in the wall of human and bovine arteries which undergo spontaneous hydroxyapatite calcification in tissue culture (9), and which we term calcifying vascular cells (CVC).<sup>1</sup> We have now characterized calcifying vascular cells as a unique subpopulation of artery wall cells which are capable of exhibiting several of the phenotypic features of bone-forming osteoblasts in vitro. In addition, the behavior of calcifying vascular cells can be modulated by certain mediators known to exist in atherosclerotic arteries.

These results suggest that calcifying vascular cells may play a role in atherosclerotic calcification. Furthermore, calcifying vascular cells offer a unique opportunity to study arterial calcification in vitro, and they may ultimately offer another target at which to direct anti-atherosclerotic therapies.

## Methods

**Cell cultures.** Bovine aortic endothelial cells (BAEC) were isolated by collagenase digestion, as previously described (10), and clones were established by limiting dilution. Bovine aortic smooth muscle cells (BASMC) were isolated by outgrowth from medial explants from thoracic aortas as previously described (9). Calcifying vascular cells were isolated from cultures of BASMC in which multicellular nodules spontaneously appeared. From these nodule-forming cultures, cells were cloned by limiting dilution and single cell harvesting. Clonal lines were identified as CVC by their positive staining with monoclonal antibody 3G5 (11), and by their ability to form calcified nodules in tissue culture. The standard tissue culture media for all cell types was Dulbecco's Modified Eagle's medium (DME) (Irvine Scientific, Santa Ana, CA) with 15% fetal bovine serum (Hyclone Laboratories; Logan, UT) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 U/ml), and fungizone (0.25  $\mu$ g/ml) all from Irvine Scientific.

**Indirect immunofluorescence.** Cells were grown in two-well chamber slides (Costar; Pleasanton, CA), fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO), and incubated for 1 h with fluorescein isothiocyanate-conjugated antibodies against CD2 and CD11c, diluted 1:100. Immediately after staining, the cells were washed and then examined by fluorescence photomicroscopy (Zeiss Axioscop 20; Carl Zeiss Inc., Thornwood, NY).

**Immunostaining.** Fresh specimens of human abdominal aorta, thoracic aorta, and carotid endarterectomy were obtained from the UCLA Medical Center renal transplant, cardiac transplant and vascular surgery services. Specimens were frozen in OCT compound (Tissue Tek, Miles Inc., Elkhart, IN), 5- $\mu$ m cryosections were prepared, and the slides were post-fixed in 4% paraformaldehyde. Mouse monoclonal antibody (mAb) 3G5 directed against a surface ganglioside present on CVC was used either as undiluted hybridoma supernatant (American Type Culture Collection, Rockville, MD) or as purified antibody diluted 1:100 (kindly provided by Dr. Ramesh Nayak, Boston, MA).

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1. Abbreviations used in this paper: BAEC, bovine aortic endothelial cells; BASMC, bovine aortic smooth muscle cells; BMP-2a, bone morphogenetic protein 2a; CVC, calcifying vascular cells.

Mouse monoclonal anti-BMP-2a (h3b2/17.8.1, kindly provided by Genetics Institute, Inc., Cambridge, MA) was used at a dilution of 1:100 and rabbit polyclonal antibody to human osteopontin (LF-7, kindly provided by Dr. Larry Fisher, National Institutes of Health, Bethesda, MD) was used at a dilution of 1:300. Specimens were first incubated with a blocking solution containing 1.5% (vol/vol) nonimmune goat serum. Primary antibody was applied for 2 h, followed by application of biotinylated secondary antibodies for 30 min. Endogenous peroxidase activity was quenched with 0.3% H<sub>2</sub>O<sub>2</sub> before incubation with the avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA). The reaction was developed using 3-amino-9-ethyl carbazole (Sigma Chemical Co.) and specimens were counterstained with hematoxylin (EM Diagnostic Systems, Gibbstown, NJ).

**Characterization of the 3G5 epitope.** The binding specificity of mAb 3G5 was characterized using the procedure of Ravindranath et al. (12). Polystyrene microtiter wells (Falcon, Lincoln Park, NJ) were coated with 4  $\mu$ g each of a panel of glycolipids consisting of lactosyl ceramide, galactosyl ceramide, asialo-GM<sub>1</sub>, GM<sub>1</sub>, GM<sub>2</sub>, GM<sub>3</sub>, GD<sub>3</sub>, GD<sub>1a</sub>, GD<sub>1b</sub>, and GT<sub>1b</sub> (all from Sigma Chemical Co.). The wells were treated with 4% human serum albumin (Hyland-Baxter, Glendale, CA), followed by addition of 100  $\mu$ l of mAb 3G5 (1:100) to each well. The plates were incubated for 1 h at 37°C then washed with 0.1% Tween-20 (Sigma Chemical Co.) in PBS. Antibody binding was assessed with peroxidase-coupled goat anti-mouse IgM (1:1,000) using ortho-phenylenediamine dihydrochloride as the substrate. The absorbance at 490 nm was measured in a kinetic microplate reader (UV Max; Molecular Devices, Menlo Park, CA). To assess ganglioside affinity of mAb 3G5 after periodate oxidation, antigen-coated plates were first treated with 100  $\mu$ l of 0.04 M periodic acid for 30 min at 37°C.

**TGF- $\beta$  treatment of endothelial cells.** Cloned BAECs were plated sparsely into two-well chamber slides and cultured in standard media. After 48 h, the cells were washed with PBS and treated with media containing 1 ng/ml TGF- $\beta$ 1. Media was changed every 3 d. Cells were tested by immunofluorescent staining with mAb 3G5 at 4, 7, and 21 d of treatment.

**Northern analysis.** RNA was isolated using the guanidinium thiocyanate method (13) from confluent monolayers of cells grown in 15% fetal bovine serum. Equal amounts of RNA were loaded onto a 1% agarose and formaldehyde gel, separated, then transferred to a nylon membrane (Zeta-Probe Blotting Membranes; BioRad Laboratories, Richmond, CA) and fixed with 0.05 N NaOH. The membranes were prehybridized for 1 h in buffer and hybridized for 18 h at 55°C using cDNA probes for human osteopontin (kindly provided by Dr. Marion Young, National Institute of Dental Research, Bethesda, MD) and human osteonectin (ATCC), labeled with [<sup>32</sup>P]dCTP (ICN, Irvine, CA). The membranes were washed, dried and exposed to film (3M Corporation, St. Paul, MN) at -70°C.

**Western analysis.** Phenol red-free DME (GIBCO BRL, Gaithersburg, MD) without serum was conditioned on confluent cell monolayers for 72 h, centrifuged at 14,000 g for 15 min, then either concentrated with Centriprep® concentrator (Amicon Corp., Beverly, MA) or lyophilized and reconstituted with deionized water. Cell extracts were prepared from confluent monolayers by scraping into 20 mM Tris-HCl buffer, with 0.2% aprotinin, sonication, and centrifugation at 14,000 g for 15 min. Cell lysates and conditioned media proteins were separated on a 7% SDS-polyacrylamide gel, (Novex X-Cell Mini-Cell, San Diego, CA) loaded with equal amounts of protein per lane and transferred onto polyvinylidene difluoride membranes (Immobilon-P Transfer Membrane; Millipore Corp., Bedford, MA) using an electrophoretic transfer cell (Trans Blot; Bio Rad Laboratories). Immunoblotting was performed using mouse anti-human osteonectin (Haematologic Technologies Inc., Essex Junction, VT), and rabbit anti-bovine collagen type I (Chemicon International, Temecula, CA) diluted 1:100, detected with avidin-biotin-horseradish peroxidase (HRP-streptavidin; Zymed, South San Francisco, CA) and 4-chloro-1-naphthol (Sigma Chemical Co.).

**Alkaline phosphatase detection.** Cells were grown on two-well chamberslides and fixed in 4% paraformaldehyde. Alkaline phosphatase activity was demonstrated in cultured cells by incubating the slides with 5-bromo-4-chloro-3-indolyl-phosphate,4-toluidine salt (Boehringer Mannheim, Indianapolis, IN). Secreted alkaline phosphatase activity was assayed in phenol red-free DME without serum, conditioned on confluent monolayers for 72 h, by measuring release of p-nitrophenol from p-nitrophenyl phosphate (Alkaline phosphatase assay; Sigma Diagnostics). Secreted alkaline phosphatase activity was normalized for total protein content as determined by the dye binding method of Bradford (Bio-Rad Protein Assay).

**Osteocalcin assay.** Cell supernatants were collected from confluent monolayers incubated in culture medium with 5% fetal calf serum for 72 h. Secreted osteocalcin was quantified by radioimmunoassay for bovine osteocalcin (INCSTAR, Stillwater, MN) with duplicate measurements performed for each sample. The assay was checked for reproducibility in validation tests consisting of triplicate assays on four different CVC clones. The mean standard deviation for the four different clones was 5.7% of the mean osteocalcin value. The background osteocalcin level (that present in the serum) was assessed by performing the assay using media conditioned by U2OS cells—an osteosarcoma line known to lack osteocalcin secretion (14). Osteocalcin content was normalized for total protein.

**Growth factor treatment of CVC.** Calcifying vascular cells were plated sparsely into two-well chamber slides to delay spontaneous nodule formation. After 72 h, media was replaced with media containing either 5 ng/ml of platelet-derived growth factor-BB (PDGF-BB; R&D Systems, Minneapolis, MN), 1000 U/ml of interferon- $\gamma$  (IFN- $\gamma$ ; Endogen Inc., Boston, MA), or 1 ng/ml TGF- $\beta$ 1 (R&D Systems). The media was renewed every 3 d.

**Sterol treatment of CVC.** Calcifying vascular cells, BAEC, and BASMC were plated on 6-well tissue culture plates (Costar Corp., Cambridge, MA) at a density of 100,000 cells/well in standard media. After 48 h, this media was replaced with media containing either 1  $\mu$ g/ml of 25-hydroxycholesterol (Sigma Chemical Co.), 10<sup>-7</sup> M 1,25-dihydroxyvitamin D<sub>3</sub> (BIOMOL Research Labs, Inc., Plymouth Meeting, PA), or vehicle alone. All sterols were diluted in 100% ethanol to a final concentration of 0.1% ethanol. The media was changed every 3 d for a total of 24 d, and the nodules in each well were counted under phase contrast microscopy.

## Results

Nodule-forming cells were obtained from six of six bovine aortas harvested. Approximately 80 clones were derived from three of these aortas, and 20–40% of the clones formed calcified nodules. The nodule-forming clones were defined as CVC.

**Table I. Immunocytochemical Profiles of Vascular Wall Cells in Culture**

	SM actin	NonSM actin	Factor VIII	CD11c	CD2	mAb 3G5
Endothelial Cells	-	+	+	n.d.	n.d.	-
Smooth Muscle Cells	+	-	-	n.d.	n.d.	-
Monocytes	-	-	-	+	-	-
Lymphocytes	-	-	-	-	+	-
Fibroblasts	-	+	-	n.d.	n.d.	-
Pericytes	+	+	-	n.d.	n.d.	+
Calcifying Vascular Cells	+	+	-	-	-	+

n.d., not determined.

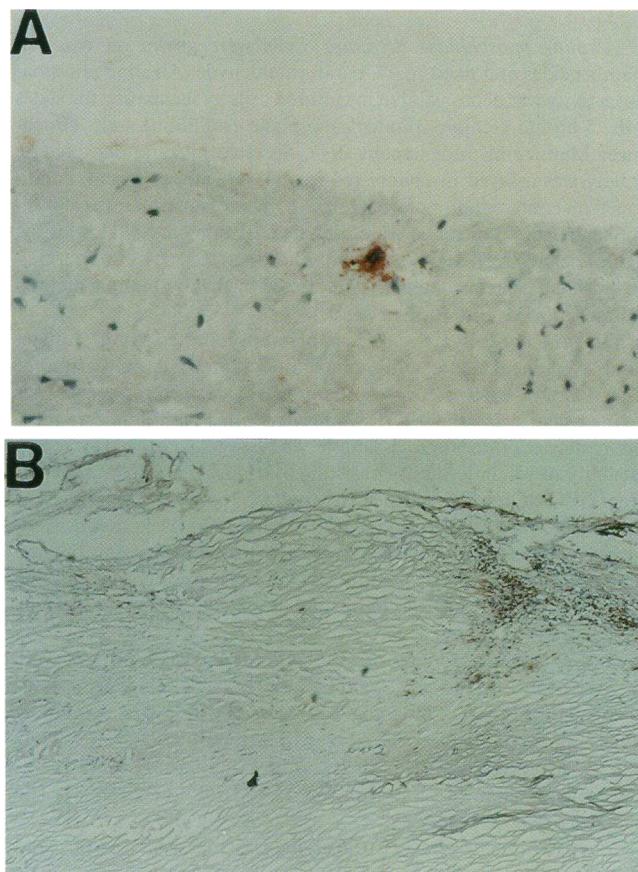


Figure 1.

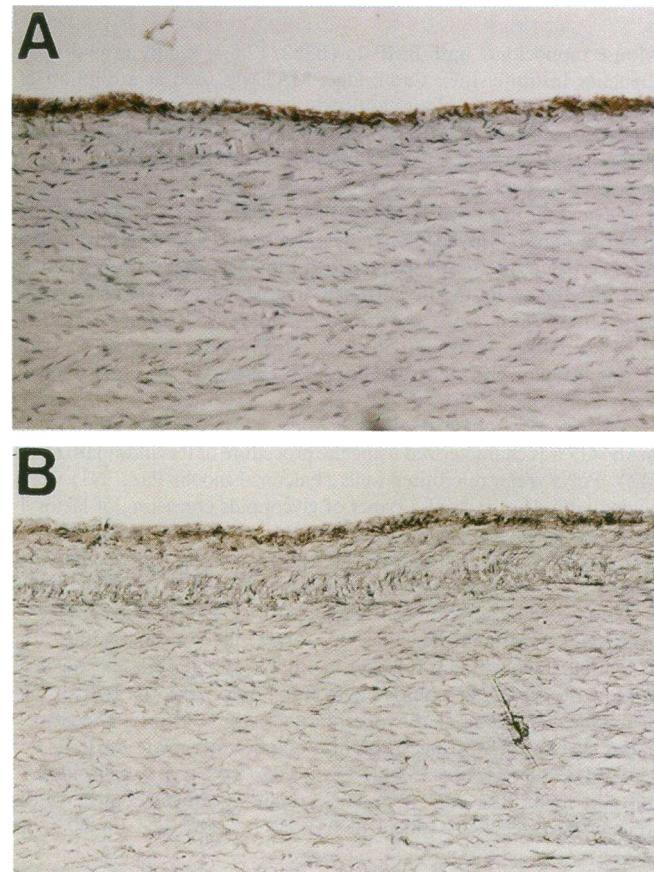


Figure 2.

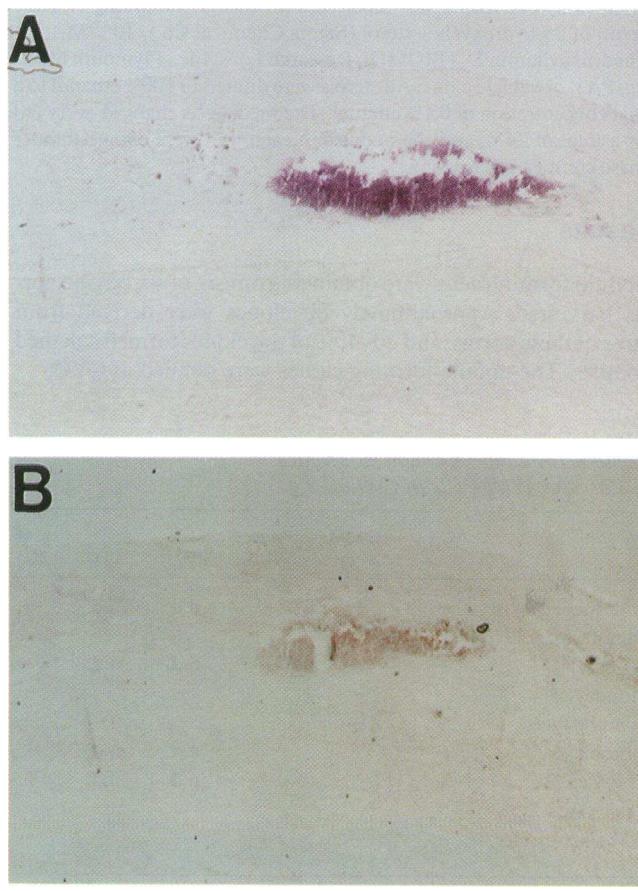


Figure 3.

Figure 1. Pattern of 3G5 positivity in aortic wall. The majority of 3G5 positive cells were found in the aortic intima in a variety of different patterns. (A) Sparse pattern of immunostaining in a frozen section of human abdominal aorta ( $\times 25$ ). (B) Focal pattern in a frozen section of human abdominal aorta with a mild intimal lesion ( $\times 4$ ).

Figure 2. Colocalization of 3G5 with osteopontin in human abdominal aortic intima. (A) Diffuse pattern of 3G5 staining. (B) Sequential section stained with anti-osteopontin antibody. (C) Sequential section stained with irrelevant antibody (negative control) shows no staining.

Figure 3. Colocalization of BMP-2a with calcification in human aortic intimal lesion. (A) Hematoxylin and eosin staining shows calcification in purple. (B) Sequential section stained with anti-BMP-2a antibody.

CVC were characterized immunocytochemically using markers for cells present in or able to migrate into the vessel wall. CVC did not stain with antibodies directed against CD2 and CD11c, indicating their distinction from typical lymphocytes and monocytes. They also did not stain for Factor VIII-related antigen indicating their distinction from endothelial cells. They did, however, stain positively for  $\alpha$ -smooth muscle actin,  $\beta$ -nonsmooth muscle actin, and monoclonal antibody 3G5 (9). This immunocytochemical profile of CVC is identical to that of microvascular pericytes (9, 15), but distinct from the profiles of normal BASMC, BAEC, fibroblasts, lymphocytes and monocytes (Table I).

To determine whether this surface marker was expressed on cells *in vivo*, we used mAb 3G5 to stain frozen sections of human arteries. One section from each of 10 human arterial specimens was examined, including seven abdominal aortas, one thoracic aorta, and two carotid endarterectomy specimens. All sections examined contained 3G5 positive cells, and these were located primarily in the intima rather than the media (479 vs 77 cells). The pattern of 3G5 staining varied greatly from single isolated cells, to small clusters, to diffuse intimal staining (Figs. 1 and 2A). All specimens also exhibited adventitial staining, in a pattern consistent with pericytes known to exist in the capillaries of the *vasa vasorum* (16).

Frozen sections were also stained for osteopontin, a bone-related phosphoprotein, and bone morphogenetic protein-2a. Osteopontin staining often colocalized with 3G5 staining (Fig. 2). BMP-2a staining colocalized with calcification (Fig. 3), however it did not appear to colocalize with 3G5, suggesting that this epitope may be lost as mineralization occurs.

Nodule formation by the CVC preceded mineralization, and in all clones tested, mAb 3G5-positivity was correlated with nodule formation. To assess the nature and possible role of the 3G5 epitope in arterial calcification, characterization of this molecule was undertaken. Nayak and colleagues previously reported that mAb 3G5 recognizes a ganglioside that migrates on thin layer chromatography midway between GM1



Figure 5. Alkaline phosphatase activity in calcifying vascular cells. Photomicrograph of histochemical staining showing alkaline phosphatase activity concentrated in nodule containing calcifying vascular cells.  $\times 25$ .

and GM2 (17). Thus, we tested 3G5 for its ability to bind to a panel of known gangliosides and related glycolipids. mAb 3G5 bound most avidly to the ganglioside GM3 (sialyl-lactosylceramide; NeuAc $\alpha$   $\rightarrow$  3Gal $\beta$ 1  $\rightarrow$  4Glc $\beta$ 1  $\rightarrow$  Cer) (Fig. 2); it did not bind to glycolipids lacking the sialic acid side chain. Binding of mAb 3G5 to GM3 was enhanced approximately twofold by periodate oxidation (Fig. 4), which cleaves unsubstituted 1,2 glycals in the glycerol side chain of sialic acid (18).

Alkaline phosphatase activity was identified in cloned CVC and in the cell supernatant. Calcifying vascular cells in sparse monolayer had only faint staining for alkaline phosphatase activity. Those in nodules, however, demonstrated intense staining for alkaline phosphatase activity (Fig. 5). Secreted alkaline phosphatase was also demonstrated in the supernatant of all CVC clones assayed, and the level of alkaline phosphatase was significantly greater in cultures producing nodules compared with those without nodules ( $3.87 \pm 0.93$  vs.  $2.28 \pm 0.40$  U/mg protein;  $P < 0.05$ ).

The bone-specific protein osteocalcin was identified in several CVC clones assayed. The level of osteocalcin varied from 0.24 to 3.21 ng/mg protein, with a background level of 0.038 ng/mg protein. This variability in osteocalcin level is a feature consistent with the known phenotypic heterogeneity among clonal osteoblastic lines (19).

Messenger RNA for the protein osteonectin was detected in CVC cultures by Northern analysis (Fig. 6A), and the protein was detected in both CVC lysate and conditioned medium by Western analysis (Fig. 6B). The level of osteonectin produced by CVC was approximately equivalent to the level produced by BASMC, as measured by densitometry. The CVC lysate, however, displayed two bands on Western blotting, one which migrated with the osteonectin band seen in the BASMC and one which migrated at a slightly higher molecular weight. In the supernatant, however, only the higher molecular weight band was present, suggesting posttranslational modification of osteonectin by the CVC with secretion of only the modified form. The presence of two osteonectin bands has previously been described in cultured osteoblasts (20, 21).

Messenger RNA for osteopontin, a factor involved in binding of cells to bone mineral, was detected in all CVC clones tested by Northern analysis (Fig. 6C). No osteopontin mRNA

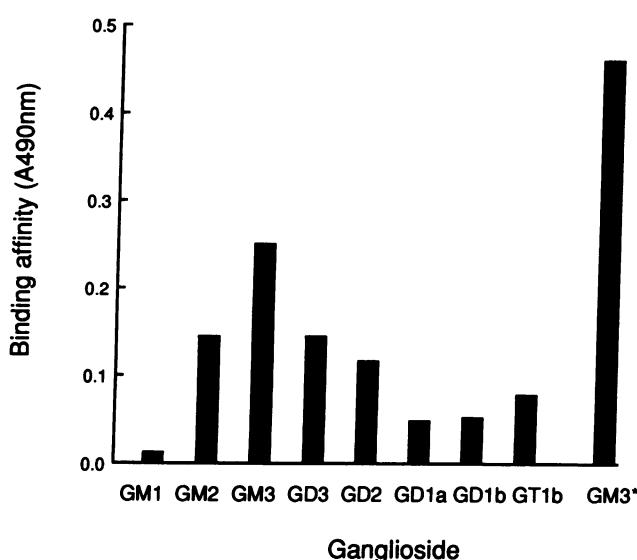
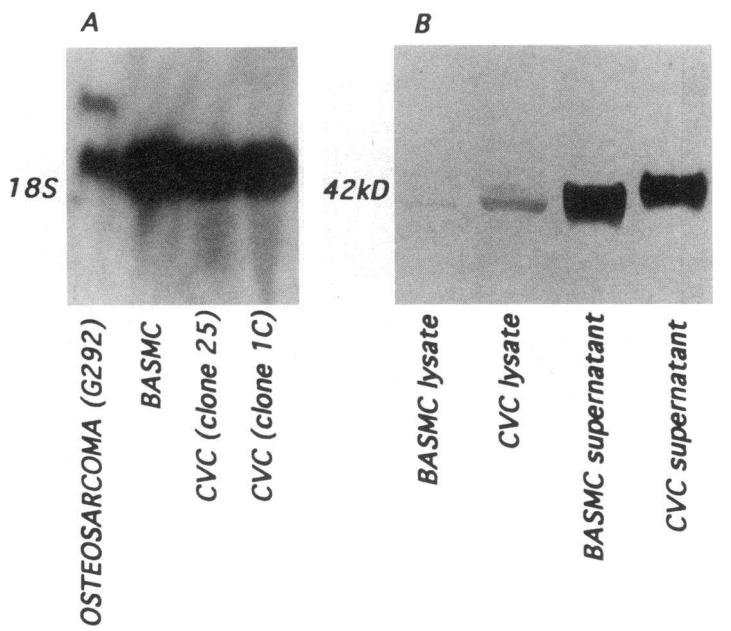


Figure 4. Binding affinity of mAb 3G5 to specific gangliosides. Affinity for gangliosides of defined structure, including periodate-treated ganglioside GM3 (signified as GM3\*).



**Figure 6.** Expression of bone related proteins in calcifying vascular cell clones. (A) Northern analysis of CVC and BASMC for osteonectin. (B) Western analysis of CVC and BASMC cell lysates and supernatants for osteonectin. (C) Northern analysis of CVC and BASMC for osteopontin.

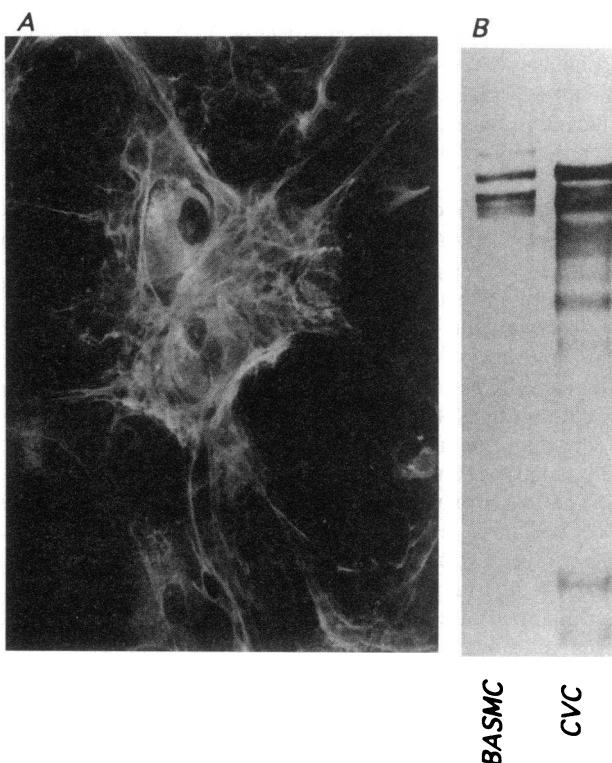
was detected in cloned BASMC, however a very faint band for osteopontin mRNA was detected in non-cloned BASMC. This is apparently due to production of osteopontin by some but not all BASMC as other authors have described (22, 23), whereas all CVC clones tested produced mRNA for osteopontin.

Collagen type I, the primary collagen in bone, was identified in CVC by immunofluorescence (Fig. 7 A) and secreted

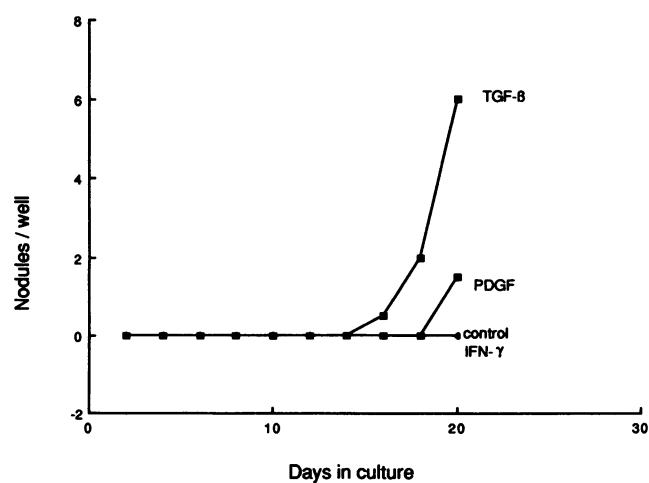
type I collagen was detected in CVC supernatants by Western analysis (Fig. 7 B). Collagen type I was detected in BASMC supernatants as well, but at a level 3–10 times lower than in CVC.

When grown in media supplemented with TGF- $\beta$ 1, CVC dramatically increased their rate of nodule formation. No increase in nodule formation was seen with IFN- $\gamma$ , and only a small increase was seen with PDGF-BB (Fig. 8).

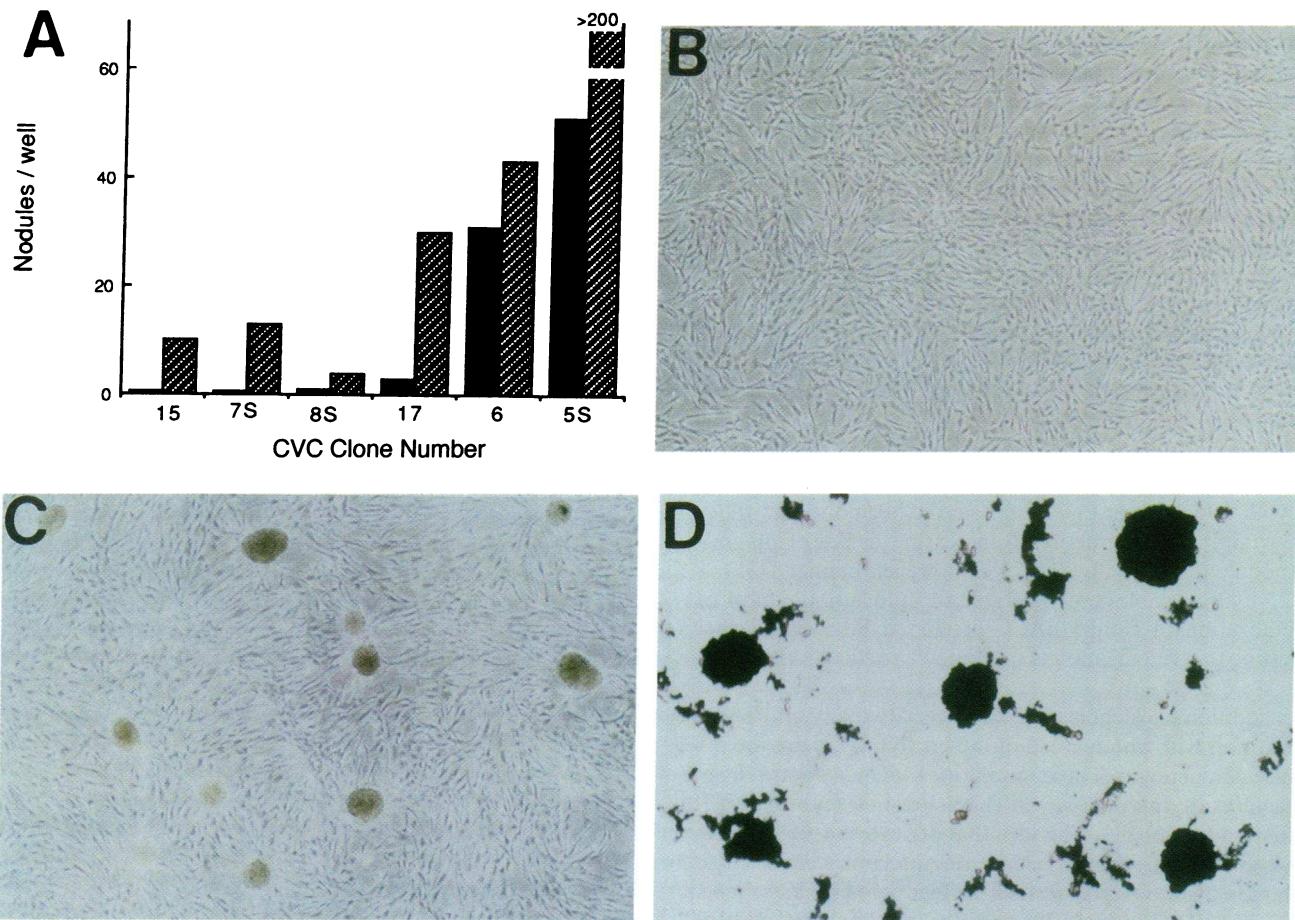
Despite this significant effect on CVC nodule formation, TGF- $\beta$ 1 did not induce nodule formation in other cell-types such as BAEC. However, TGF- $\beta$ 1 was unexpectedly found to modulate expression of the 3G5 epitope in BAEC. After treatment with TGF- $\beta$ 1, an increasing fraction of BAEC became



**Figure 7.** Collagen I production by calcifying vascular cell clones. (A) Immunofluorescence of CVC for collagen type I.  $\times 20$ . (B) Western analysis of CVC and BASMC supernatants for collagen type I.



**Figure 8.** Effect of cytokines on nodule formation by calcifying vascular cells. Nodules were counted in sparsely seeded cultures for 20 d after continuous treatment with PDGF (PDGF; 5 ng/ml), interferon- $\gamma$  (IFN- $\gamma$ ; 1,000 U/ml), or transforming growth factor- $\beta$  (TGF- $\beta$ ; 1 ng/ml). Nodule formation was accelerated with TGF- $\beta$  and PDGF. IFN- $\gamma$  did not increase nodule formation compared with control cultures.



**Figure 9.** Effect of 25-hydroxycholesterol on nodule formation by calcifying vascular cells. (A) Bar graph showing control CVC (closed bars) and 25-hydroxycholesterol treated CVC (cross-hatched bars) for six different CVC clones. (B) Phase contrast photomicrographs of control CVC treated with vehicle alone ( $\times 4$ ) and (C) treated with 1  $\mu$ g/ml 25-hydroxycholesterol ( $\times 4$ ). (D) Von Kossa stain of 25-hydroxycholesterol treated CVC ( $\times 10$ ), showing intense calcification of the nodules.

mAb 3G5 positive, being evident in some at 4 d of treatment, and in 30–50% at 21 d. Despite being 3G5 positive however, the TGF- $\beta$ 1 treated BAEC did not form nodules, even after more than 30 d in culture.

When grown in media supplemented with 25-hydroxycholesterol, nodule formation by CVC was increased approximately sixfold (ranging from a 1.5 to greater than a 10-fold increase) in six of six clones tested (Fig. 9). These nodules were highly calcified as demonstrated by von Kossa staining (Fig. 9D). No increase in CVC nodule formation was induced by vehicle alone ( $n = 13$ ), 1,25-dihydroxyvitamin D<sub>3</sub> induced either no increase ( $n = 4$ ) or a minimal increase ( $n = 4$ ) in nodule formation (data not shown). Neither 1,25-dihydroxyvitamin D<sub>3</sub> nor 25-hydroxycholesterol induced nodule formation in BASMC or in BAEC ( $n = 3$ ).

## Discussion

Atherosclerotic calcification is a widely recognized, yet poorly understood process. The prevailing view has been that this calcification occurs by passive precipitation of calcium mineral, is not regulated, and contributes little to the pathogenesis of clinical coronary artery disease. These ideas, however, have recently been changing, due in part to new imaging techniques

which allow the widespread prevalence of coronary calcification to be appreciated (1, 24, 25) and the recognition that calcification is associated with adverse clinical events (2–6, 26).

We hypothesize that atherosclerotic calcification is similar to osteogenesis, based on the morphologic similarities between advanced arterial calcification and trabecular bone (7), the presence of matrix vesicles in atherosclerotic lesions (8), and the presence of bone morphogenetic protein-2a, one of the most potent osteogenic factors known, in atherosclerotic plaques (9). Given this hypothesis, the present investigation was aimed at understanding some of the cellular and molecular mechanisms of atherosclerotic calcification, in particular the cells that produce the mineralizing matrix, and the factors present in atherosclerotic plaques that mediate the process.

To identify potential artery wall cells producing calcium mineral, we isolated from cultures of BASMC a subpopulation of CVC. By immunocytochemistry, CVC are distinct from other cell types known to exist in the aortic wall, they are large and stellate, they produce extensive extracellular material, and they readily form multicellular nodules which undergo spontaneous calcification. These cells exhibit many features characteristic of osteoblasts including production of alkaline phosphatase, collagen type I, osteocalcin, osteonectin and osteopontin (27, 28, 29), suggesting similarities to osteogenesis.

In examining possible factors in atherosclerotic plaques that might induce CVC to express osteoblastic features, two factors had the most consistent and dramatic effects on the formation of mineralized nodules: TGF- $\beta$ 1 and the oxysterol 25-hydroxycholesterol. TGF- $\beta$ 1, which is secreted by osteoblasts (30), has been suggested to be a major regulator of bone cell function (31, 32), and is found in high levels in atherosclerotic plaques (33); it significantly increased the rate of mineralized nodule formation by CVC and induced expression of the 3G5 epitope on endothelial cells. Despite 3G5 positivity however, treated-BAEC did not form nodules or calcify, indicating that, despite being in an environment suitable for CVC mineralization, the BAEC were apparently not the appropriate cellular substrate for osteoblastic differentiation.

25-hydroxycholesterol, one of the oxidized sterols found in atherosclerotic plaques (34, 35), consistently increased the rate of mineralized nodule formation by CVC. This effect was not seen with vehicle alone or with the steroid hormone 1,25-dihydroxyvitamin D<sub>3</sub>. Despite greatly increasing the rate of mineralized nodule formation by the CVC, however, neither TGF- $\beta$ 1 nor 25-hydroxycholesterol was able to induce nodulation or mineralization in cloned BAEC or cloned BASMC, further suggesting that calcification is specific to a particular precursor cell.

In addition to osteoblasts, CVC also share many similarities in common with another cell of mesenchymal origin, the microvascular pericyte. These similarities include morphologic characteristics, in vitro growth characteristics, and mAb 3G5 positivity (11, 36, 37). The idea that pericytes or stellate pericyte-like cells may play a role in atherosclerosis has been set forth previously. Over 20 years ago Geer noted stellate-shaped cells in the aortic intima (38) and Reckter, Orekhav, and colleagues described stellate cells in the aortic intima which they felt resembled pericytes in ultrastructure and periendothelial location (39, 40). Diaz-Flores and colleagues have suggested that pericytes are responsible for the intimal thickening of atherosclerosis (41), and Nayak and colleagues noted that mAb 3G5, which identifies pericytes, stained aortic intimal cells (11).

Few cells exist in the adult organism which retain pluripotentiality. The pericyte is considered to be one (42, 43). Pericytes have been reported to be precursors of several mesenchymal cell types, including osteoblasts (44, 45) and smooth muscle cells (46). We speculate that calcifying vascular cells may represent one step along the mesenchymal lineage which is intermediate between that of pericytes and osteoblasts. Calcifying vascular cells may also relate to the "mesenchymal intimal cells" identified by Wilcox and colleagues (47) and the "primitive mesenchymal cells" described by others.

The final question we consider here is the possible origin of the calcifying vascular cells. In situ immunostaining of normal appearing human and bovine aortas show mAb 3G5 positive cells in the intima, and mAb 3G5 positive cells were found in primary cultures of BASMC. In addition, cloned BASMC and cloned CVC exhibit separate and distinct phenotypes in vitro which do not interconvert with standard culture conditions. These observations suggest that CVC are not merely an artifact of culture conditions, but rather a specific population of the artery wall. The presence of CVC in morphologically normal arteries suggests that they are not a phenotypic modulation in response to injury or disease, however the possibility that they are in some way derived from smooth muscle cells or other indigenous intimal cells cannot be excluded. Migration of peri-

cytes of the adventitial vasa vasora is another possible origin of CVC, as is migration into the intima of cells from the peripheral circulation. At present there is insufficient data to distinguish among these possibilities.

We suggest that calcifying vascular cells are a mesenchymally-derived subpopulation of intimal cells which are capable of exhibiting osteoblastic potential in vitro. It is possible that under the appropriate conditions and under the influence of mediators present in atherosclerosis, these cells may express this osteoblast-like phenotype in vivo as well. And ultimately, modifying calcifying vascular cell behavior may influence some of the clinical outcomes of atherosclerosis.

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