American Journal of Pathology, Vol. 149, No. 1, July 1996
Copyright © American Society for Investigative Pathology

Autocrine Secretion of Osteopontin by Vascular Smooth Muscle Cells Regulates Their Adhesion to Collagen Gels

Andrea S. Weintraub,* Cecilia M. Giachelli,† Robert S. Krauss,‡ Manuela Almeida,† and Mark B. Taubman§

From the Department of Pediatrics, the Department of Biochemistry,‡ the Department of Medicine,§ Cardiovascular Institute,§ and Brookdale Center for Molecular Biology,§ Mount Sinai School of Medicine, New York, New York, and the Department of Pathology,† University of Washington, Seattle, Washington*

Osteopontin (OPN) is a secreted protein postulated to facilitate vascular smooth muscle cell (VSMC) adhesion and migration. Rat aortic VSMC lines were isolated after infection with recombinant retroviruses harboring OPN sense and antisense constructs. All lines grew normally in monolayer culture. On three-dimensional collagen gels, normal VSMCs and lines containing sense constructs (n = 15) or empty vector (n = 10) attached to the gel and invaded the matrix. Four of five antisense clones did not adhere or invade. Antisense clones had lower OPN levels after stimulation with angiotensin II than sense clones or clones containing the empty vector (antisense, 257 ± 102 ng/ml; sense, 473 ± 104; vector, 434 ± 66). Non-adhering antisense clones had lower mean OPN levels after angiotensin II stimulation (161 ± 47 ng/ml) than sense or antisense lines with normal adhesion (486 ± 63 ng/ml). The ability to adhere correlated with OPN levels >250 ng/ml. Adhesion and invasion were fully restored with addition of 100 to 200 ng/ml of exogenous OPN and were inhibited in normal VSMCs by incubation with 1 μg/ml anti-OPN antibody. The autocrine secretion of OPN appears to play an important role in VSMC adhesion, spreading, and invasion. (Am J Pathol 1996, 149:259–272)

Vascular smooth muscle cells (VSMCs) are the predominant cell type in the media of the mammalian artery. Normal VSMCs provide structural support to the vessel, contribute to the maintenance of vascular tone, and exist in a quiescent state with a differentiated contractile phenotype. Vascular injury induces modulation of VSMCs from a quiescent to a proliferative state characterized by cell division, cell migration into the intima, and secretion of extracellular matrix. This sequence of events is characteristic of the intimal hyperplasia seen in atherosclerosis[1–4] and has been implicated in the pathogenesis of vessel restenosis after balloon angioplasty of human coronary arteries.[5–7]

The extracellular matrix may play an integral role in mediating the migratory and proliferative responses of VSMCs.[8] Many investigators have examined VSMCs cultured in three-dimensional (3D) gels and have determined that matrix composition profoundly influences cell adhesion, growth, and migration.[9–11] The matrix may provide the framework upon which growth factors, cytokines, and chemoattractants can adhere and come into proximity with VSMCs. The matrix may also provide a structural milieu that can either promote or inhibit cell adhesion, migration, and growth. VSMCs are likely to play an important role in determining the composition of their surrounding extracellular matrix by secreting a variety of matrix and matrix-degrading proteins, including collagen,[12–15] elastin,[16] tenascin,[17,18] thrombospondin,[17,19,20] fibrillin,[21] collagenase,[22]

Supported by National Institutes of Health grants HL43302 (M. B. Taubman), HL18645 (C. M. Giachelli), DK47659 (C. M. Giachelli), and CA59474 (R. S. Krauss), by a Sinsheimer Scholar's Award (R. S. Krauss), and by a Career Scientist Award (M. B. Taubman) from the Irma T. Hirschl-Monique Weill-Caulier Charitable Trusts. A. S. Weintraub is a recipient of National Institutes of Health National Research Service Award HL08888.

Accepted for publication February 29, 1996.

Address reprint requests to Dr. Mark B. Taubman, Box 1269, Mt. Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

and metalloproteinases.[23,24] This autoregulatory secretion of matrix by VSMCs may create the requisite environment for VSMC adhesion, growth, and migration.

Osteopontin (OPN) is a secreted, phosphorylated glycoprotein that belongs to the family of extracellular matrix proteins containing arginine-glycine-aspartate (RGD) sequences. These sequences are recognized by adhesion-promoting cell surface receptors and facilitate cell-to-substrate and cell-to-cell attachment.[25] In bone, OPN is thought to be multifunctional by mediating matrix mineralization by osteoblasts and matrix degradation by osteoclasts. It is through the mediation of osteoclast cell attachment that OPN mediates bone resorption.[26,27] Many non-osteogenic tissues, including kidney, sensory epithelium of the embryonic ear, placenta, decidua, lung, and neural tissue synthesize OPN, as do a variety of mammalian fibroblastic and epithelial tumor cells.[28] It has been suggested that OPN is involved in angiotensin II (Ang)-induced tubulointerstitial nephritis,[29] in fetal-maternal interactions at the level of the placenta,[28,30,31] and in modulation of nitric oxide synthesis.[32,33] OPN has also been found in the plasma and sera of patients with advanced metastatic colon and ovarian carcinomas and has been implicated in the development of malignancy and enhancement of metastasis.[32,34–36] The extra-osteogenic secretion of OPN suggests that it plays an important role beyond bone mineralization.

A vascular role for OPN has recently been suggested. Giachelli et al[37,38] identified OPN in a rat pup VSMC cDNA library differentially screened with cDNA prepared from cultured pup and adult aortic VSMCs. OPN mRNA levels were found to be higher in the cultured pup VSMCs than in the adult cells, suggesting a role in vascular development. Another recent report[39] correlated the induction of OPN with the entry of VSMCs into the cell cycle. We also identified OPN in a differential screen targeted to identify platelet-derived growth factor (PDGF)-responsive genes in rat aortic VSMCs.[40,41] In addition to its regulation in VSMC culture, OPN mRNA levels were increased fivefold within 48 hours of aortic balloon injury and remained elevated for 7 days. *In situ* hybridization localized the induction of OPN to the injured media and developing neointima.[38]

The function of OPN in the vessel wall is not yet known. A recent report[42] demonstrated that OPN protein could affect VSMC adhesion and chemotaxis in a modified Boyden chamber. Based on these findings, OPN has been postulated to be involved in the adhesive interactions required for migration of VSMCs from the media into the intima after vascular injury. To further explore this hypothesis, we established retrovirally infected stable cell lines containing OPN cDNA in the sense and antisense orientations. Inhibition of endogenous OPN secretion with an antisense construct blocked VSMC adhesion, spreading, and invasion in 3D collagen gels. Attachment and invasion were restored by the addition of exogenous OPN protein to the culture media and were blocked in normal VSMCs in the presence of anti-OPN antibody. These data suggest that VSMC adhesion and invasion may be in part regulated via an autocrine mechanism involving OPN secretion.

Materials and Methods

Cell Culture

VSMCs were isolated from the thoracic aortas of 200- to 300-g male Sprague Dawley rats by enzymatic dissociation as previously described.[43] Cells were grown in 100-mm plates containing Dulbecco's modified Eagle medium (DME) supplemented with 10% heat-inactivated calf serum (CS), 100 U/ml penicillin, and 100 mg/ml streptomycin and serially passaged before reaching confluence. To produce quiescence, cells were incubated in a defined media composed of DME supplemented with 1 μmol/L insulin, 5 μg/ml transferrin, 0.2 mmol/L L-ascorbic acid, and 25 μg/ml bovine serum albumin (BSA) for 48 hours. VSMCs infected with OPN constructs were maintained in DME supplemented with 10% CS and 200 μg/ml hygromycin B initially (Sigma Chemical Co., St. Louis, MO); the hygromycin concentration was decreased to a maintenance dose of 50 μg/ml after individual clones were isolated. All cell lines retained a stable phenotype after >20 passages in monolayer culture.

Isolation of Cell Lines and Stable Transfectants

The full-length cDNA encoding rat OPN was cloned in sense or antisense orientations into the *Not*I site of the retroviral vector pMV12.[44] pMV12 contains the 5′ and 3′ long terminal repeat sequences of the Moloney murine sarcoma virus and the hygromycin phosphotransferase gene driven by the herpes simplex-thymidine kinase promoter. ψ2 packaging cells were plated at a density of 5×10^5 cells/100-mm plate and transfected with 20 μg/plate of pMV12-OPN sense or antisense constructs, as well as with the empty vector, using calcium phosphate.[45] Transfected ψ2 cells were split into selective media con-

taining 200 μg/ml hygromycin. Healthy colonies were selected, pooled, and reseeded, and at approximately 75% confluence, cells were refed with nonselective media. After 24 hours, the viral supernatant was collected, centrifuged at 1000 rpm at 4°C, filtered through 0.2-μm filters, and frozen at −80°C.

Thirteenth passage proliferative VSMCs were plated at a density of 5×10^5 cells/100-mm plate 24 hours before infection. VSMCs were exposed to 3 ml of viral supernatant with 8 μg/ml polybrene (Sigma) for 4 hours; control plates were exposed to DME/10% CS plus polybrene. Approximately 48 hours after infection, cells were split into hygromycin-containing media. Clones were selected by ring cloning, expanded, and frozen at −80°C.

Measurement of OPN mRNA

Isolation of total RNA from VSMCs, agarose gel electrophoresis, and transfer to nitrocellulose were performed as previously described.[40] [^{32}P]UTP-labeled (800 Ci/mmol; Amersham, Arlington Heights, IL) OPN sense and antisense riboprobes of equal size were synthesized[46] to a specific activity of 10^8 cpm/mg and used at 10^6 cpm/ml. Prehybridization and hybridization were performed as previously described[40] except that final washes were in 0.1X standard saline citrate (1X SSC = 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate at 80°C.[46] Equal loading of total RNA was verified by ethidium bromide staining of the 18S and 28S ribosomal RNA. Autoradiograms were scanned using a Silverscanner (Lacie, Beaverton, OR) and Adobe Photoshop 2.5.1 software (Adobe Systems, Mountain View, CA). Two-dimensional densitometry, using 256 gray scales, was done using Image 1.43 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). All measurements were normalized to the 18S ribosomal RNA.

Measurement of OPN Protein

Media samples were collected from VSMCs after 48 hours of incubation in defined media and from quiescent VSMCs 24 hours after treatment with 1 μmol/L Ang. Samples were used to coat the bottom of 96-well immunoabsorbent plates (Nunc Maxisorp, Naperville, IL). Nonspecific binding was blocked by incubating wells with phosphate-buffered saline (PBS) containing 10% normal horse serum and 2% bovine serum albumin for 2 hours. Wells were rinsed with PBS and incubated for 1 hour with a 1:2500 dilution of MPIIIB10$_1$ anti-OPN antiserum. Reagents from a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), prepared according to the manufacturer's protocol, were utilized for the remaining detection steps. Wells were rinsed with PBS, incubated with a biotinylated horse anti-mouse antibody for 1 hour, rinsed again, and then incubated with preformed avidin/biotinylated horseradish peroxidase complexes for 1 hour. After a final rinse, wells were incubated with a developing solution containing the chromogenic substrate *0*-phenylenediamine dihydrochloride. Color development was quenched by adding 100 μl of 4.5 mol/L H_2SO_4. OPN concentrations were determined by comparison to a standard curve generated with rat VSMC-derived OPN purified as previously described.[42] Protein concentrations were determined using a micro BCA assay kit (BioRad, Hercules, CA).

Determination of Cell Growth

VSMCs containing OPN sense and antisense constructs as well as the empty vector were plated in monolayer in 12-well culture dishes at a density of 5×10^4 cells per well in DME/10% CS. Cells were harvested by trypsinization and centrifugation, and total cell numbers for each clone were obtained by counting triplicate aliquots with a Coulter counter (Coulter Electronics, Hialeah, FL). Cell numbers were plotted as the fold increase in cell number over time zero on a logarithmic scale.

Preparation of 3D Collagen Gels

The 3D collagen matrices were constructed in 12-well culture plates using a modification of previously described methods.[10,11] Vitrogen 100 (Celtrix, Santa Clara, CA), a solution of pepsin-solubilized bovine dermal collagen, was mixed with 1X and 10X M199 medium (Gibco, Grand Island, NY) in a ratio of 3.5:1 as previously described,[11] for a final collagen concentration of 2 mg/ml, and was titrated to pH 7.4. The gels were allowed to polymerize overnight at 37°C and then rinsed three times with DME/10% CS before use.

Cell Adhesion Assays

Normal VSMCs and VSMCs containing OPN sense and antisense constructs, as well as the empty vector, were grown to confluence in 100-mm plates and trypsinized at 37°C. Cell suspensions were centrifuged at 600 × *g* at 4°C, and pellets were resuspended in a minimal volume of DME/10% CS for quantification of cell number. Approximately 5×10^4

cells were plated per well of the collagen matrix in DME/10% CS. Replicate experiments were performed after plating cells in monolayer in 12-well culture dishes at a density of 5×10^4 cells per well in DME/10% CS. The culture medium was removed from the wells at 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, and 3 hours after seeding, and the gels or plastic wells were washed vigorously with PBS. Gels were photographed, and cell number in the media and PBS washes was quantified by automated counting.

Cell Invasion Assays

Normal VSMCs and VSMCs containing OPN sense and antisense constructs, as well as the empty vector, were grown to 70% confluence in 100-mm plates and trypsinized at 37°C. Cell suspensions were centrifuged at 1000 rpm at 4°C, and pellets were resuspended in a minimal volume of DME/10% CS for quantification of cell numbers. Approximately 5×10^4 cells were plated per well of the collagen matrix in DME/10% CS. Cell invasion was assessed by phase contrast microscopy with a 10× objective at 1 hour, 6 hours, 24 hours, and 4 days after seeding. At each time point, four random fields in each well were inspected, and the number of cells on and 100 μm below the gel surface was determined.

Two-Dimensional Cell Migration Assay

VSMCs were grown to confluence on 60-mm plates. Following the method of Irving and Lala,[47] monolayers were first rinsed with PBS, and then using a sterile razor blade to demarcate a midline wound, cells were scraped from one-half of the plate. Duplicate plates were rinsed twice with PBS and examined under the microscope to ensure complete cell removal. After 72 hours, cells were counted by setting a 0.1-mm^2 calibrated ocular grid against two preselected areas per plate. For both sense- and antisense-infected VSMCs, measurements were made of the number of migrant cells (± SEM) within the grid, as well as the maximal distance of migrant cells from the initial wound.

Results

Isolation of Cell Lines Stably Infected with OPN Constructs

Rat aortic VSMCs were infected with recombinant retroviruses containing OPN constructs to create cell lines stably underexpressing OPN. A retroviral expression vector (pMV12) containing a full-length cDNA encoding rat OPN in the sense or antisense orientations was used with the $\psi 2$ packaging system[44,48] to generate defective Moloney murine sarcoma virus particles, which were then used to infect rat aortic VSMCs. pMV12 encodes the selectable marker gene *hygro*, thus allowing isolation of infected cell clones that co-express the OPN cDNA. VSMCs were also infected with the pMV12 vector lacking a cDNA insert. Fifteen clones containing OPN in the sense orientation, five clones containing OPN antisense construct, and ten clones containing the empty vector were isolated and expanded through hygromycin resistance. These clones were tested for adhesion and invasion in 3D collagen gels. All VSMC clones infected with either the OPN-sense construct or the empty vector adhered and invaded the 3D gels normally, whereas four of five clones infected with the OPN-antisense construct did not display normal adhesion or invasion. Based on this initial observation, all five antisense clones were chosen for more extensive evaluation, with four sense clones and two clones containing the empty vector selected as controls. Studies with these clones are described as follows.

Cell Morphology and Growth in Monolayer Culture and 3D Gels

On plastic monolayers, all VSMC lines studied had the typical spindle-shaped appearance of uninfected VSMCs and grew as usual in a hill and valley pattern (not shown). Using a wound and scrape migration assay on the plastic monolayers,[47] approximately equal numbers of sense (64 ± 9 per 0.1-mm^2 grid) and antisense cells (65 ± 6) migrated from the wound to mean distances of 8.9 and 8.8 mm, respectively (sense, range 4 to 15 mm; antisense, range 5 to 12 mm). These lines also demonstrated the same rate of growth on plastic (Figure 1). In contrast, there were marked differences when the clones were plated on 3D collagen gels. Representative photomicrographs of the infected VSMC lines and uninfected control VSMCs maintained in 10% CS in 3D collagen gels are shown in Figure 2.

Adhesion and invasion of VSMCs into the 3D matrices was essentially an all-or-none phenomenon. When plated on 3D gels, uninfected VSMCs and VSMCs harboring the empty vector quickly attached to and invaded the gel to form lattice-like networks throughout the matrix (Figure 2, A and D). Most sense clones invaded and formed cell connections

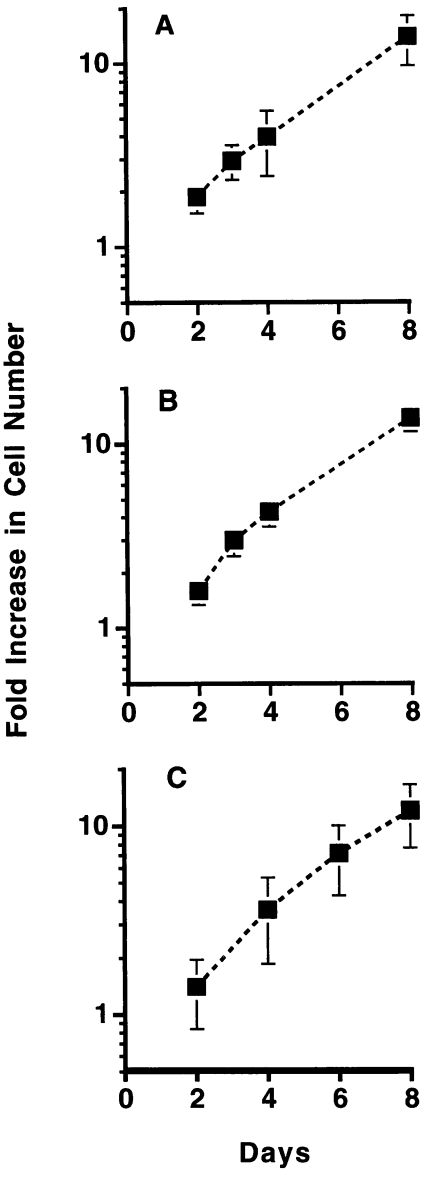

Figure 1. *Growth of VSMC clones in monolayer culture. Clones were grown in 12-well plates in DME/10% CS, and cell counts were obtained on the days indicated. Curves represent the average of four VSMC clones infected with antisense OPN cDNA* (**A**), *four VSMC clones infected with sense OPN cDNA* (**B**), *and four VSMC clones infected with the empty vector* (**C**), *plotted as the fold increase in cell number over time zero on a logarithmic scale. Vertical lines represent standard deviation.*

with a pattern indistinguishable from the controls. A few sense lines also formed larger cell clusters (Figure 2C); this phenotype was seen only with the sense clones. There was no correlation between secreted OPN levels and the formation of these cell nodules. In contrast, four of five clones that harbored the antisense cDNA construct failed to adhere to or invade the gels or to form networks, even after 4 days (Figure 2B).

Measurement of OPN mRNA Levels in VSMC Clones

To ascertain that OPN antisense mRNA was expressed by putative antisense-infected VSMCs, RNA blot analysis was performed using a riboprobe synthesized from the full-length OPN cDNA insert. As demonstrated in Figure 3A, expression of high levels of OPN antisense mRNA was detected in all four non-adhering, non-invading antisense clones. There were no differences in antisense mRNA levels between quiescent and Ang-stimulated antisense clones, demonstrating that the retroviral promoter was not Ang responsive. Ang was selected as the agonist to test OPN expression because it is the most potent inducer of OPN in normal VSMCs.[38] No antisense mRNA was seen in untransfected VSMCs or VSMCs transfected with retroviruses containing OPN in the sense orientation or with the empty retroviral vector. In contrast, the one antisense clone that adhered and invaded normally expressed minimal levels of antisense mRNA.

To examine OPN mRNA expression in the selected clones, RNA blot analysis was also performed on replicate blots using a full-length antisense riboprobe. As shown in Figure 3B, OPN cRNA hybridized to an mRNA species of ~1.5 kb. Baseline and stimulated OPN mRNA levels were in general similar among clones harboring the sense cDNA, antisense cDNA, or empty vector. Of note, larger mRNA species were also detected in some of the sense clones.

Measurement of OPN Protein Levels in VSMC Clones

To assess levels of OPN secretion in selected clones, an enzyme-linked immunosorbent assay was performed on the culture medium of quiescent cells after 24 hours of Ang stimulation. As shown in Figure 4A, antisense clones had lower OPN levels (± SEM) after maximal stimulation than sense clones or clones containing the empty vector (antisense, 257 ± 102 ng/ml; sense, 473 ± 104; empty vector, 434 ± 66). Of note, the sole antisense clone that adhered and invaded normally had a stimulated OPN level of 644 ng/ml. The antisense clones displaying impaired adhesion and invasion had much lower mean OPN levels after Ang stimulation (161 ± 47 ng/ml) than the normally adhering and invading clones (486 ± 63 ng/ml). These data suggest that the retroviral system was successful in generating stable antisense clones with inhibited OPN secretion.

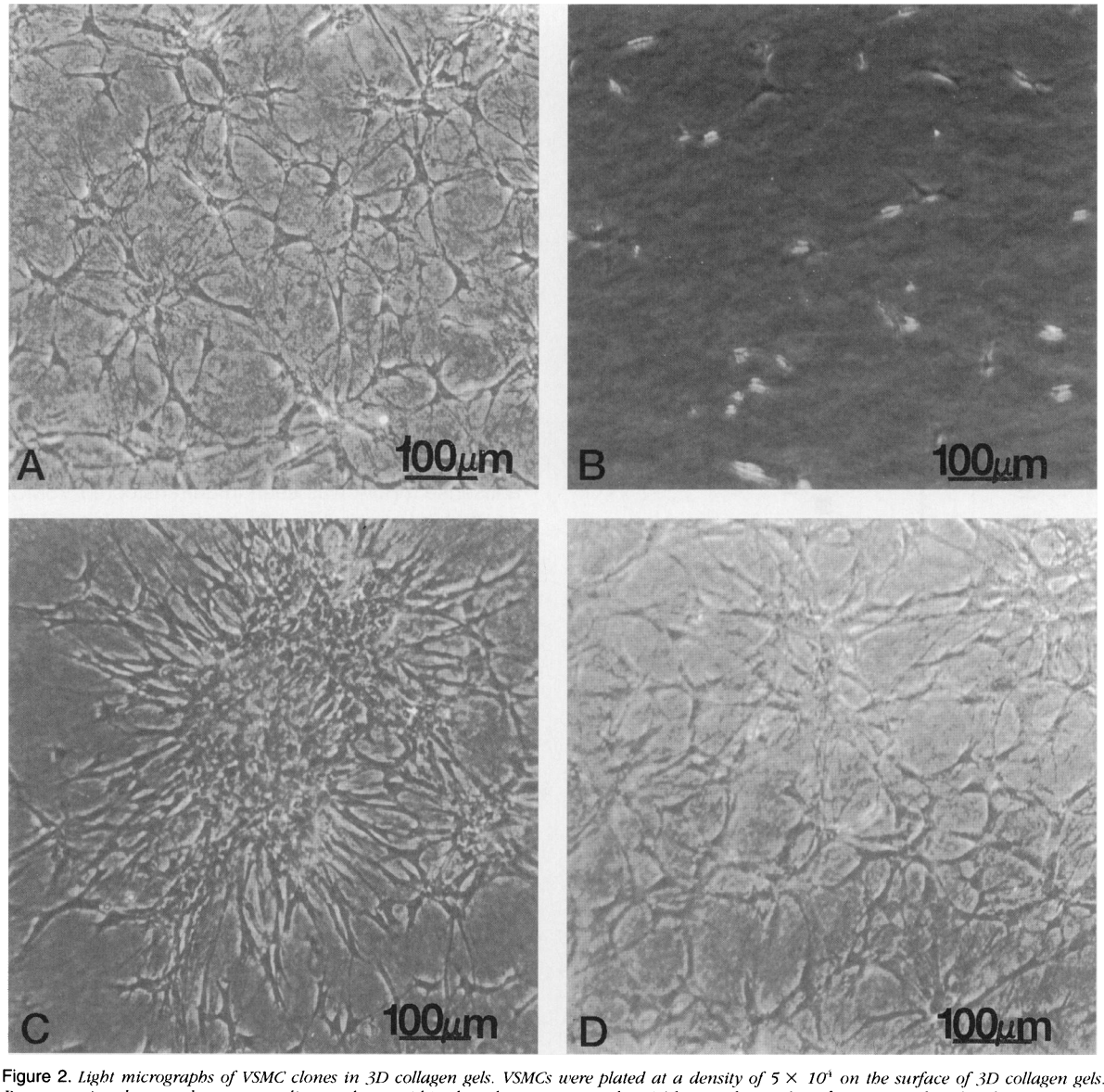

Figure 2. *Light micrographs of VSMC clones in 3D collagen gels. VSMCs were plated at a density of 5 × 10^4 on the surface of 3D collagen gels. Representative photographs corresponding to planes within the gel matrix were taken 24 hours after seeding.* **A:** *VSMCs infected with empty vector.* **B:** *VSMCs infected with antisense OPN cDNA.* **C:** *VSMCs infected with sense OPN cDNA.* **D:** *Normal VSMCs. Each VSMC line and the uninfected control VSMCs were plated in triplicate and examined on at least two different occasions.*

Correlation of OPN mRNA to Protein Levels in VSMC Clones

To examine the relationship between Ang-stimulated OPN mRNA expression and the secretion of OPN protein, the ratio of OPN sense/antisense mRNA, as determined by two-dimensional densitometry, was plotted against OPN protein levels for the five antisense clones (Figure 4B). The four non-adhering, non-invading antisense clones had sense/antisense mRNA ratios of <1 and secreted OPN protein levels of <236 ng/ml. In contrast, the single antisense clone with a sense/antisense mRNA ratio of 5 had a protein level of 644 ng/ml and exhibited normal adhesion and invasion.

As a second approach to examining the relationship between Ang-stimulated OPN mRNA expression and OPN secretion, the level of OPN antisense mRNA was subtracted from the level of sense mRNA and the result plotted against OPN protein levels (Figure 4C). Negative values, corresponding to clones with higher levels of antisense than sense mRNA, were seen only in the four non-adhering, non-invading antisense clones and were associated

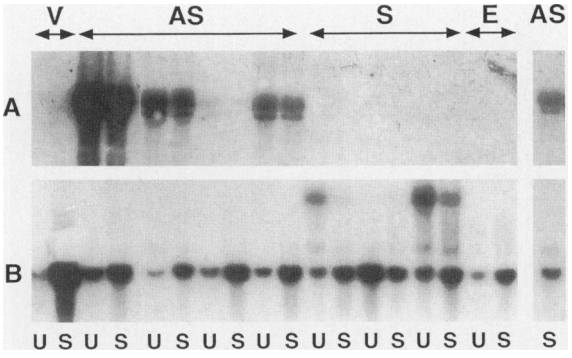

Figure 3. *RNA blot analysis of OPN expression in infected VSMC lines. Total RNA (10 μg/lane) was isolated from unstimulated quiescent VSMC lines (U) and from quiescent VSMC clones after 24 hours of stimulation (S) with 1 μmol/L Ang. V, uninfected VSMCs; AS, OPN antisense VSMC clones; S, OPN sense VSMC clones; E, VSMCs infected with empty vector. Replicate blots were hybridized with [^{32}P]UTP-labeled sense* (A) *and antisense* (B) *OPN riboprobes as described in Materials and Methods. Equal loading of total RNA was verified by ethidium bromide staining of the 18S and 28S ribosomal RNA.*

with low levels of OPN protein. All clones (sense, antisense, and empty vector) with normal adhesion and invasion in 3D gels had positive values (ie, higher levels of sense mRNA than antisense) and were associated with higher levels of OPN protein. It should be noted that these experiments were performed on duplicate blots, using sense and antisense probes of the same size and specific activity and hybridized, washed, and autoradiographed under identical conditions.

Restoration of VSMC Adhesion and Invasion by Exogenous OPN

The use of antisense cDNAs, although effective in decreasing OPN levels, could be preventing attachment and invasion into collagen gels by an unrelated mechanism. To test the specificity of the constructs, antisense clones were suspended in culture medium in the presence of exogenous OPN protein at concentrations of 50, 100, 200, and 400 ng/ml and plated onto the collagen matrices. Exogenous OPN protein restored the ability of these clones to adhere, spread, and invade the gel in a dose-dependent fashion (Figure 5). Normal adhesion and invasion were seen with all antisense clones at exogenous OPN concentrations between 100 and 200 ng/ml. Exogenous OPN protein had no effect on the behavior of the normally adhering and invading antisense clone.

Inhibition of VSMC Adhesion and Invasion by Anti-OPN Antibody

As an additional test of the specificity of the antisense constructs, normal VSMCs were suspended in culture medium in the presence of OP199, a neutralizing goat polyclonal antibody against rat smooth-muscle-cell-derived OPN,[42] at concentrations of 400, 800, and 1000 ng/ml, and plated onto the collagen matrices. Adhesion and invasion of normal VSMCs on collagen gels was blocked at an antibody concentration of 1 μg/ml (Figure 6A) but was unaffected by incubation with non-immune IgG (Figure 6B). Assuming the molecular weight of a prototype IgG antibody at ~150,000 and OPN at ~66, this effect was seen at an approximate 1:1 ratio of OPN to antibody on a molecule-per-molecule basis.

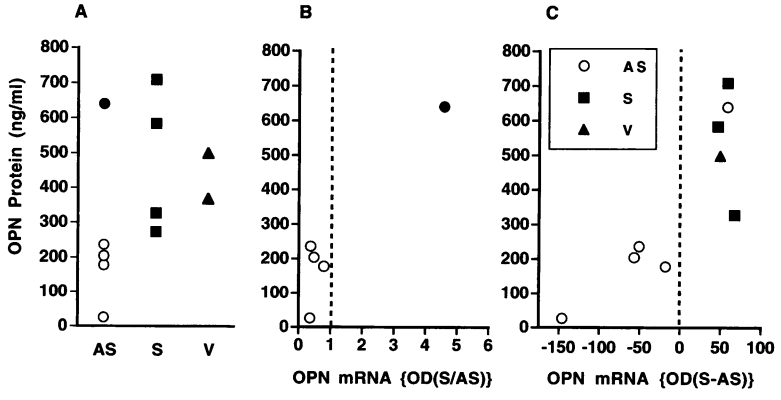

Figure 4. *Correlation of OPN protein secretion with mRNA expression.* A: *Measurement of OPN protein secretion by infected VSMC lines. Quiescent VSMC lines infected with antisense (AS, ○) or sense OPN cDNA (S, ■), or with the empty vector alone (V, ▲) stimulated with 1 μmol/L Ang for 24 hours. The conditioned media samples were harvested and OPN levels were measured by ELISA as described in Materials and Methods. The closed and open shapes represent invasive and non-invasive clones, respectively.* B: *Ratio of OPN sense/antisense mRNA* versus *OPN protein levels. The ratio of OPN sense/antisense mRNA (seen in Figure 3), as determined by two-dimensional densitometry, was plotted against OPN protein levels for the five antisense clones. The closed and open shapes represent invasive and non-invasive clones, respectively.* C: *OPN mRNA (sense-antisense)* versus *OPN protein levels. The level of OPN antisense mRNA was subtracted from the level of sense mRNA and the result was plotted against OPN protein levels. Negative mRNA values correspond to clones with higher levels of antisense than sense mRNA. The closed and open shapes represent invasive and non-invasive clones, respectively.*

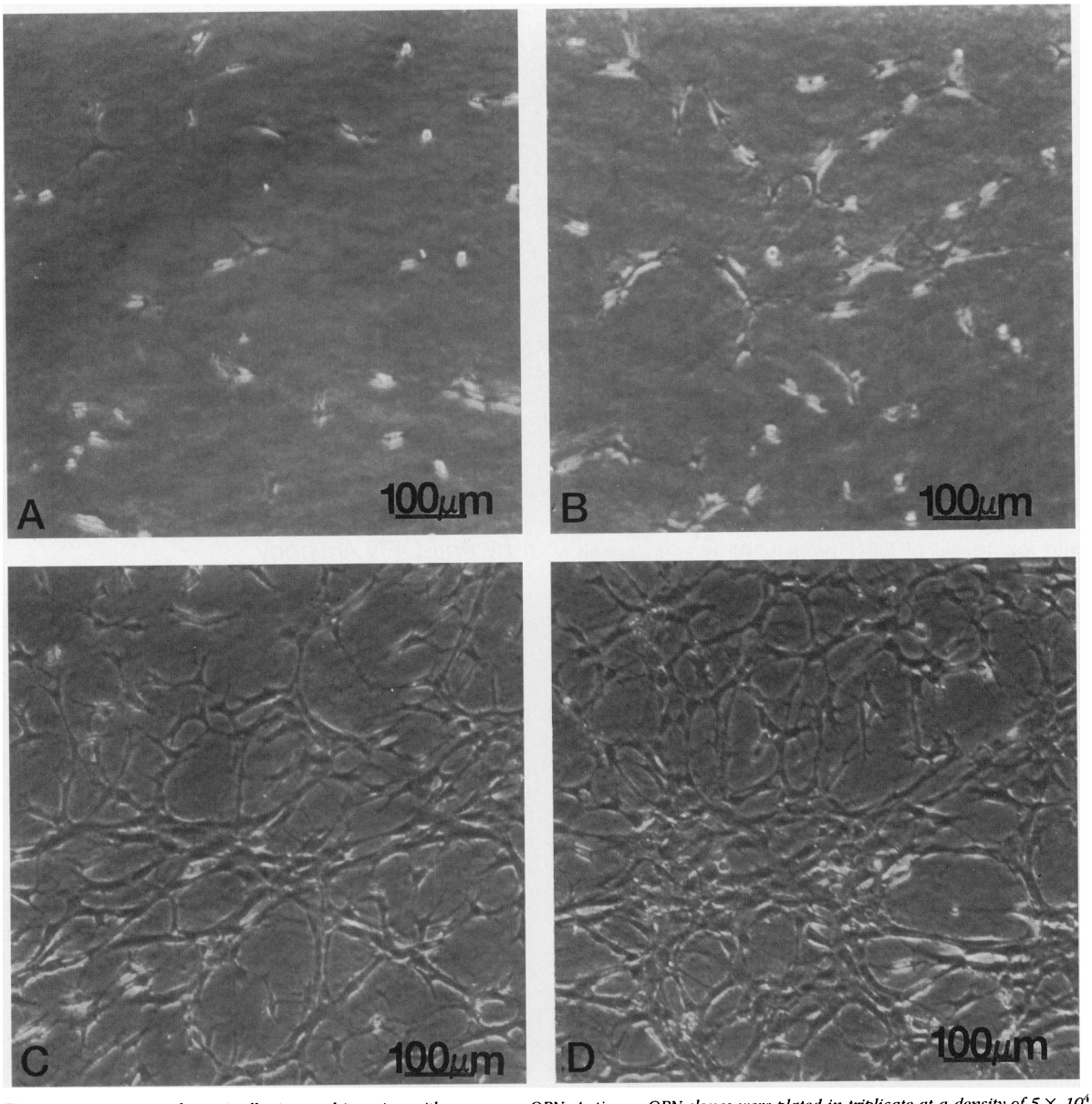

Figure 5. *Restoration of VSMC adhesion and invasion with exogenous OPN. Antisense OPN clones were plated in triplicate at a density of 5 × 10^4 on the surface of 3D collagen gels in the presence of increasing concentrations of exogenous OPN.* A: *No OPN.* B: *OPN, 50 ng/ml.* C: *OPN, 100 ng/ml.* D: *OPN, 400 ng/ml. For these studies, all four antisense lines were examined. Photographs corresponding to planes within the gel matrix were taken 24 hours after seeding and are representative of the four lines.*

Role of Adhesion in OPN-Mediated Invasion

As a first step in determining the mechanism underlying the failure of antisense clones to invade collagen gels, VSMC adhesion and spreading were examined during the initial 3 hours after seeding. By light microscopy, 30 minutes after seeding, apparently equal numbers of uninfected VSMCs (Figure 7A) and antisense (Figure 7E) cells were present on the surface of the gel. Within 1 hour, uninfected VSMCs had begun to spread and invade the surface of the gel (Figure 7B), although the majority of antisense cells had failed to spread or invade (Figure 7F). Within 2 to 3 hours, uninfected VSMCs had spread and begun to invade into the gel (Figure 7, C and D). There were no differences in the spreading or invasion between the uninfected VSMCs, sense clones, or clones containing the empty vector. In contrast, the number of antisense cells remaining on the gel surface was greatly reduced (Figure 7, G and H).

To further study the early events after seeding, cell counts were performed on the culture medium at 15-minute intervals during the first hour after seeding, and then hourly during the next 2 hours. At each

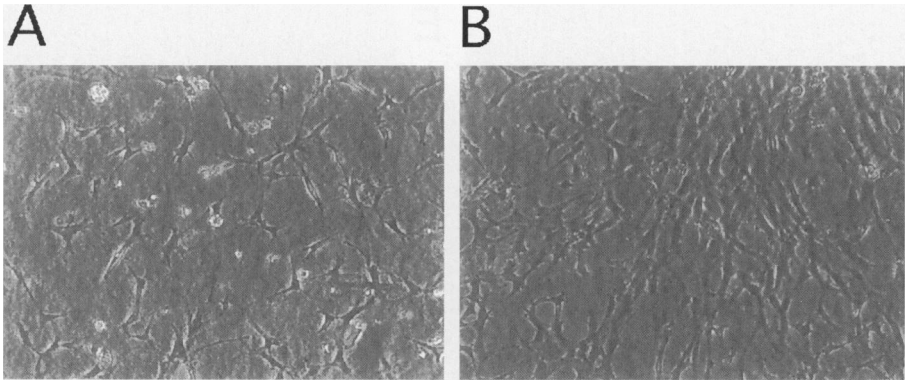

Figure 6. *Inhibition of VSMC adhesion and invasion in the presence of anti-OPN antibody. Normal VSMCs were plated in triplicate at a density of* 5×10^4 *on the surface of 3D collagen gels in the presence of increasing concentrations of OP 199, a goat polyclonal antibody against rat smooth-muscle-cell-derived OPN.* **A:** *Anti-OPN antibody, 1 μg/ml.* **B:** *Non-immune IgG, 1 μg/ml. Photographs corresponding to planes within the gel matrix were taken 24 hours after seeding.*

time point, the culture medium was removed, the gels were washed vigorously with PBS, and fresh medium was added. The cell counts on the combined media and washes from each time point are shown in Figure 8. The adhesion of antisense clones in standard monolayer cultures on plastic dishes was high, as seen in Figure 8, and identical to that seen with uninfected VSMCs, sense clones, and clones containing the empty vector (data not shown). Similarly, at 15 minutes after plating on collagen gels, equal numbers of cells were present on the gel surface (93% for uninfected VSMCs *versus* 89% for antisense). However, by the end of the first hour, approximately one-half of the antisense cells could be recovered in the media or washes, whereas most of the uninfected cells remained in the matrix (uninfected VSMCs, 84% attached *versus* 46% attached). There were no appreciable differences in the adherence of uninfected VSMCs, sense clones, and clones containing the empty vector to the collagen gels. At 3 hours after plating, few cells could be recovered from the media of either uninfected or antisense VSMCs; however, debris was present in the antisense media and washes. This debris is likely to be the remnants of non-adhering cells that were sloughed from the gel surface. These studies suggest that, although the antisense VSMCs initially adhered to the surface of the gel, they failed to spread or invade the matrix.

Discussion

VSMC proliferation and migration are pivotal processes in the development of vascular disease. The growth, migration, and morphology of VSMCs in culture have been found to be dependent in part on the components of the extracellular matrix, including fibronectin, vitronectin, laminin, hyaluronate, chondroitin sulfate, and heparin.[9–12,49–52] In this report, we demonstrate that the secretion of OPN by VSMCs affects their adhesion, spreading, and invasion into 3D collagen gels by an autocrine mechanism.

Adhesion and invasion were studied using rat aortic VSMCs stably infected with a retroviral vector containing OPN cDNA in the sense or antisense orientation. All clones had morphologies and plating and growth characteristics similar to that of uninfected VSMCs on plastic monolayers under standard cell culture conditions and did not demonstrate a transformed phenotype. Sense and antisense clones also displayed virtually identical migration behavior on plastic, both in terms of number of migrant cells and distance from origin. When plated onto 3D collagen gels, however, the antisense clones displayed distinctly different phenotypes; four out of five did not adhere, spread, or invade into the gel, whereas the attachment and invasion of sense clones and clones containing the empty retroviral vector was largely indistinguishable from normal VSMCs. These results underscore the usefulness and importance of a 3D culture system in revealing information vis-a-vis VSMC mobility and adhesion beyond that which standard two-dimensional cultures can provide. The 3D collagen gels have therefore been utilized[9–11] to better simulate the *in vivo* environment of VSMCs. Artificial matrices differ from native connective tissue in the collagen composition, in the presence of other extracellular matrix components, and in the 3D structural composition of fibrillar materials. The use of type I collagen gels has distinct advantages over other two-dimensional models. Type I collagen is a major constituent of the extracellular matrix of VSMCs *in vivo*, and VSMCs adhere rapidly to and invade into collagen gels in three dimensions, as

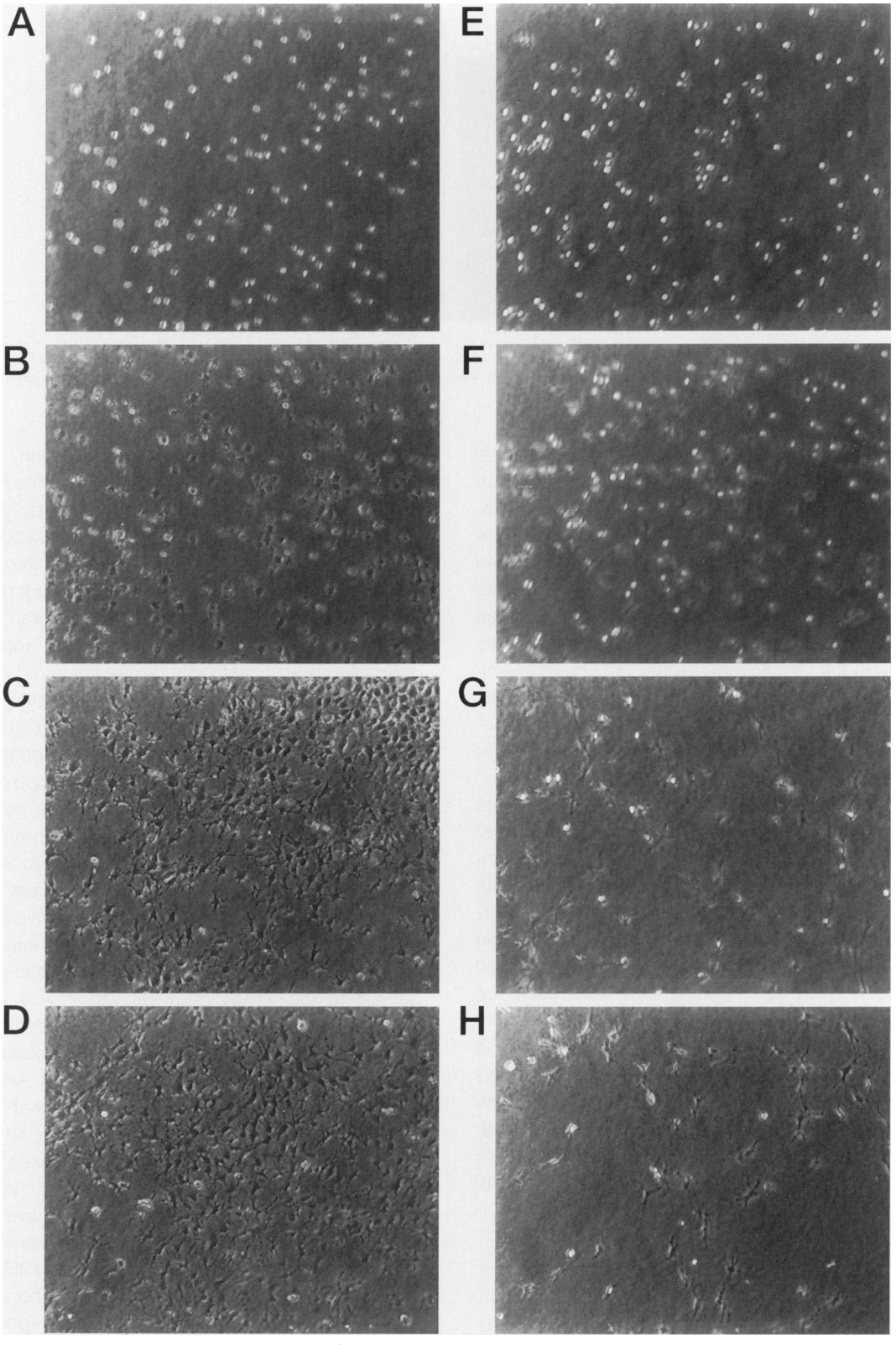

Figure 7. *Light micrographs of VSMC adhesion to 3D collagen gels. VSMCs were plated at a density of* 5×10^4 *on the surface of 3D collagen gels. Representative photographs corresponding to planes within the gel matrix were taken at intervals over the first 3 hours after plating.* **A:** *Normal VSMCs, 30 minutes.* **B:** *Normal VSMCs, 60 minutes.* **C:** *Normal VSMCs, 120 minutes.* **D:** *Normal VSMCs, 180 minutes.* **E:** *Antisense-infected VSMC clone, 30 minutes.* **F:** *Antisense-infected VSMC clone, 60 minutes.* **G:** *Antisense-infected VSMC clone, 120 minutes.* **H:** *Antisense-infected VSMC clone, 180 minutes. Antisense-infected VSMC lines and the uninfected control VSMCs were plated in duplicate and examined on at least two different occasions.*

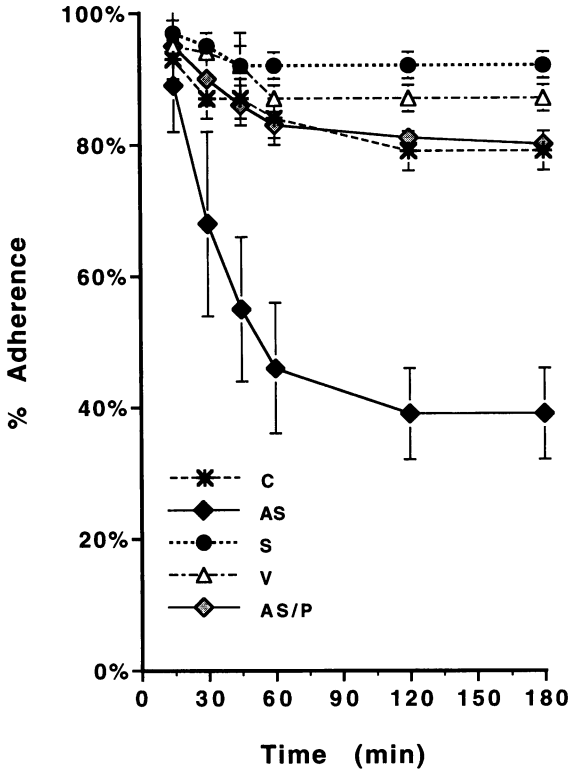

Figure 8. *VSMC adhesion to 3D collagen gels. Uninfected VSMCs (C), antisense-infected VSMCs (AS), sense-infected VSMCs (S), and VSMCs containing the empty vector (V) were plated at a density of* 5×10^4 *on the surface of 3D collagen gels. Antisense VSMCs (AS/P) were also plated at a density of* 5×10^4 *onto plastic 12-well plates. Media was removed and the gels were washed vigorously with PBS at the times indicated. Curves represent the percentage of cells remaining on the gel (ie, not recovered in the media or washes). All VSMCs were plated in duplicate and examined on at least two different occasions.*

they do *in vivo*. In addition, the gels more closely resemble the *in vivo* state by allowing greater cell-to-cell and cell-to-substrate communication during invasion.

Although the 3D system allows a more physiological look at VSMC mobility, the matrix utilized in this study consisted of collagen alone. Although it is beyond the scope of this report, it will be interesting to investigate the effect of modulating OPN levels on the invasion of VSMCs into other matrices, such as those composed of collagen in combination with RGD-containing proteins such as fibronectin or vitronectin. Immunohistochemical studies of osteoid suggest that binding of OPN to collagen may involve fibronectin, because OPN lacks a collagen attachment domain,[53] but fibronectin binds to type I collagen.[54] If OPN functions in VSMC mobility largely as an adhesive intermediate for VSMC attachment to matrix components, its effect may indeed be substrate specific. If, however, OPN acts as a VSMC ligand for the modulation of intracellular processes associated with cell adhesion, spreading, or movement, such an effect would more likely be matrix independent.

The ability of VSMCs to invade collagen gels appears to require a threshold of OPN secreted in the culture medium. The four antisense clones that attached and invaded poorly had levels of OPN protein of <236 ng/ml (mean of 161 ± 47 ng/ml) after maximal stimulation with Ang. In contrast, the only antisense clone that attached and invaded normally had an OPN level of 640 ng/ml. These were 100 to 250 ng/ml less than those found in clones containing the sense construct or the empty vector after Ang stimulation. The concept of an OPN threshold is further supported by the finding that addition of 100 to 200 ng/ml OPN to the culture medium, sufficient to raise levels to those of the adhering, invading clones, restored the normal phenotype.

The lower OPN protein levels in the poorly adhesive and invasive antisense clones could be the consequence of several phenomena. The antisense mRNA could complex with native OPN mRNA, destabilizing it and inducing its degradation. Alternatively, a stable antisense/sense complex would likely interfere with binding of OPN mRNA to ribosomes, preventing translation. Additional possibilities include inhibition of OPN transcription by the antisense mRNA or a nonspecific effect on OPN synthesis. RNA blot analysis confirmed that high levels of antisense OPN mRNA were expressed in the four clones that exhibited poor adhesion and invasion. Although there were not enough clones to establish an inverse correlation between antisense mRNA levels and OPN protein, the clone exhibiting the highest level of antisense mRNA had the lowest level of OPN protein. In contrast, the clone that adhered normally had minimal antisense mRNA and very high levels of OPN protein.

The expression of antisense mRNA did not significantly alter the steady-state levels of OPN mRNA either at baseline or upon stimulation with Ang. This suggests that the antisense mRNA did not significantly destabilize the native OPN mRNA or prevents its transcription. As shown in Figure 4B, under conditions designed to produce equivalent signals with sense and antisense riboprobes, all four antisense clones that failed to adhere and migrate properly had higher steady-state levels of antisense mRNA than sense mRNA. In addition, as shown in Figure 4C, levels of OPN protein were roughly proportional to the difference between sense and antisense mRNA. These data support the concept that the decrease in OPN protein levels in the antisense clones was due to a decrease in translation resulting from stable antisense/sense complexes.

It is worth noting that the clones containing the sense cDNA did not exhibit higher baseline or Ang-stimulated levels of the 1.5-kb OPN mRNA. In several clones, higher molecular weight species were detected by hybridization with the OPN antisense riboprobe. These species are likely to represent the presence of larger OPN transcripts formed as a consequence of integration of the retroviral construct within the genome. It is unclear whether these larger mRNAs produce functioning OPN protein. However, levels of OPN protein were not significantly greater in the sense clones than in the clones containing the empty vector or in untransfected controls. This may be due to ineffective transcription of the exogenous sense cDNA or to a negative feedback mechanism regulating endogenous OPN synthesis.

These studies do not rule out the possibility that the antisense cDNA inhibited the synthesis of a protein other than OPN that is critical to, or that the vector integrated into a site necessary for, VSMC adhesion, spreading, and invasion. The fact that four different antisense clones failed to invade normally, whereas 25 clones containing the sense or empty vector migrated normally suggests that it is not an artifact of integration. The finding that the only antisense clone that invaded normally did not express significant levels of antisense mRNA or display a reduction in OPN levels provides additional support for the specificity of the response. Most importantly, the normal phenotype was fully restored to all of the antisense clones by addition of exogenous OPN and did not require any additional factor. An additional argument in support of the specificity of the antisense effect lies in the ability of anti-OPN antibody to block normal VSMC adhesion and invasion. In this regard, it should be noted that the amount of neutralizing OPN antibody that was necessary to inhibit VSMCs in this assay (~400 ng of OPN to 1 μg of antibody) was less than that required to block the adhesion of VSMCs to OPN-coated plastic surfaces in *in vitro* assays (~13.2 ng of OPN to 1 μg of antibody[42]). This may be related to a difference in interaction of the antibody with soluble *versus* surface-bound OPN.

The mechanism underlying the involvement of OPN in VSMC invasion remains to be elucidated. In a recent report,[42] purified OPN protein was shown to be a substrate for VSMC adhesion and to be chemotactic for VSMCs in a modified Boyden chamber. These effects were inhibited by an antibody to OPN and were mediated in part through the integrin $\alpha v\beta 3$. It was hypothesized that OPN might provide a sticky surface to which VSMCs adhere and along which they migrate. The 3D culture system used in our report requires both VSMC adhesion to the surface of the collagen gel and chemokinesis of VSMCs through the gel, in part through the production of collagenases, without the need for an OPN concentration gradient.

Inhibition of OPN did not appear to prevent the initial attachment of VSMCs to the gel. Unlike normal VSMCs, antisense cells failed to spread over the gel surface and invade the matrix. These cells were ultimately sloughed from the gel surface and were recoverable in the culture medium. One explanation for this phenomenon is that the antisense lines were unable to make the correct attachments to the collagen gel to allow for spreading and invasion of the matrix. Alternatively, the primary defect in the antisense lines may be a failure to spread or invade into the gel substance, resulting in an inability to maintain attachment. In this regard, it has been proposed by Meredith et al[54] that the failure of cells to properly attach to and migrate into matrix may lead to cell death. Although it is beyond the scope of this report, it will be interesting to determine the fate of the non-adhering antisense clones through cell viability studies.

OPN mRNA and protein have recently been shown to be induced in the vessel wall after balloon arterial injury.[38] The secretion of OPN by VSMCs as an early event after vessel injury may facilitate movement of VSMCs from the media to the intima. In animal models of arterial injury, VSMC migration into the intima and subsequent proliferation are critical events in the development of intimal hyperplasia, the lesion responsible in part for vessel narrowing in atherosclerosis.[1,2] Blocking VSMC migration with antibodies to PDGF BB[3] has been successful in attenuating intimal hyperplasia in a rat carotid artery model of acute injury. OPN may provide a new target for attenuating VSMC migration. In addition, the availability of invasive and non-invasive VSMC lines may provide an exciting means for more fully elucidating the molecular mechanisms involved in vascular remodeling.

Acknowledgments

We thank Robert S. Green, M.D., for his expert assistance with statistics.

References

1. Ross R: Atherosclerosis: a problem of the biology of arterial walls and their interaction with blood components. Atherosclerosis 1981, 1:293–311

2. Clowes AW, Clowes MM, Fingerle J, Reidy MA: Regulation of smooth muscle cell growth in injured artery. J Cardiovasc Pharmacol 1989, 14(suppl 6):S12–S15
3. Ferns GAA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R: Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 1991, 253:1129–1132
4. Schwartz SM, Heimark RL, Majesky MW: Developmental mechanisms underlying pathology of arteries. Physiol Rev 1990, 70:1177–1209
5. Califf RM, Fortin DF, Frid DJ, Harlan WR III, Ohman EM, Bengston ER, Nelson CL, Tcheng JE, Mark DB, Stack RS: Restenosis after coronary angioplasty: an overview. J Am Coll Cardiol 1991, 17:2B–13B
6. Garratt KN, Edwards WD, Kaufmann UP, Vleistra RE, Holmes DR: Differential histopathology of primary atherosclerotic and restenotic lesions in coronary arteries and saphenous vein grafts: analysis of tissue obtained from 73 patients by directional atherectomy. J Am Coll Cardiol 1991, 17:442–448
7. Isner JM, Pickering JG, Weir L, Jekanowski J, Kearney MA: Proliferative activity in peripheral and coronary atherosclerotic plaque among patients undergoing percutaneous revascularization. J Clin Invest 1993, 91: 1469–1480
8. Strauss BH, Chisholm RJ, Keeley FW, Gotliev AI, Logan RA, Armstrong PW: Extracellular matrix remodeling after balloon angioplasty injury in a rabbit model of restenosis. Circ Res 1994, 75:650–658
9. Delvos U, Gajdusek C, Sage H, Harker LA, Schwartz SM: Interactions of vascular wall cells with collagen gels. Lab Invest 1982, 46:61–72
10. Wren FE, Schor AM, Schor SL, Grant ME: Modulation of smooth muscle cell behavior by platelet-derived factors and the extracellular matrix. J Cell Physiol 1986, 127:297–302
11. Boudreau N, Turley E, Rabinovitch M: Fibronectin, hyaluronan, and a hyaluronan binding protein contribute to increased ductus arteriosus smooth muscle cell migration. Dev Biol 1991, 143:235–247
12. Madri JA, Bell L, Marx M, Merwin JR, Basson C, Printz C: Effects of soluble factors and extracellular matrix components on vascular cell behavior *in vitro* and *in vivo*: models of de-endothelialization and repair. J Cell Biochem 1991, 45:123–130
13. Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA: Production of transforming growth factor β-1 during repair of arterial injury. J Clin Invest 1991, 88: 904–910
14. Snow AD, Bolender RP, Wight TN, Clowes AW: Heparin modulates the composition of the extracellular matrix domain surrounding arterial smooth muscle cells. Am J Pathol 1990, 137:313–330
15. Madri JA, Marx M: Matrix composition, organization, and soluble factors: modulators of microvascular cell differentiation *in vitro*. Kidney Int 1992, 41:560–565
16. Hinek A, Rabinovitch M: 67-kD elastin-binding protein is a protective "companion" of extracellular insoluble elastin and intracellular tropoelastin. J Cell Biol 1994, 126:563–574
17. Majesky MW: Neointima formation after acute vascular injury: role of counteradhesive extracellular matrix proteins. Texas Heart Inst J 1994, 21:78–85
18. Hedin U, Holm J, Hansson GK: Induction of tenascin in rat arterial injury: relationship to altered smooth muscle cell phenotype. Am J Pathol 1991, 139:649–656
19. Raugi GJ, Mullen JS, Bark DH, Okada T, Mayberg MR: Thrombospondin deposition in rat carotid artery injury. Am J Pathol 1990, 137:179–185
20. RayChaudhury A, Frazier WA, D'Amore PA: Comparison of normal and tumorigenic endothelial cells: differences in thrombospondin production and responses to transforming growth factor-β. J Cell Sci 1994, 107: 39–46
21. Ramirez R, Pereira L, Zhang H, Lee B: The fibrillin-Marfan syndrome connection. Bioessays 1993, 15:1–6
22. Yanagi H, Sasaguri Y, Sugama K, Morimatsu M, Nagase H: Production of tissue collagenase (matrix metalloproteinase I) by human aortic smooth muscle cells in response to platelet-derived growth factor. Atherosclerosis 1991, 91:207–216
23. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P: Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. Circ Res 1994, 75:181–189
24. Pauly RR, Passaniti A, Bilato C, Monticone R, Cheng L, Papadopoulos N, Gluzband YA, Smith L, Weinstein C, Lakatta EG, Crow MT: Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. Circ Res 1994, 75: 41–54
25. Pierschbacher MD, Ruoslahti E: Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 1984, 309:30–33
26. Oldberg A, Franzen A, Heinegard A: Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an ARG-GLY-ASP binding sequence. Proc Natl Acad Sci USA 1986, 83:8819–8823
27. Paniccia R, Colucci S, Grano M, Serra M, Vallone AZ, Teti A: Immediate cell signal by bone related peptides in human osteoclast-like cells. Am J Physiol 1993, 265: C1289–C1297
28. Nomura S, Wills AJ, Edwards DR, Heath JK, Hogan BLM: Developmental expression of 2AR(osteopontin) and SPARC(osteonectin) RNA as revealed by *in situ* hybridization. J Cell Biol 1988, 106:441–450
29. Giachelli CM, Pichler R, Lombardi D, Denhardt DT, Schwartz S, Johnson RJ: Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis. Kidney Int 1994, 45:515–524
30. Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW: cDNA cloning, mRNA distribution and heterogeneity, chromosomal location,

and RFLP analysis of human osteopontin (OPN). Genomics 1990, 7:491–502
31. Waterhouse P, Parker RS, Guo X, Lala PK, Denhardt DT: Regulated temporal and spatial expression of the calcium-binding proteins calcyclin and OPN (osteopontin) in mouse tissues during pregnancy. Mol Reprod Dev 1992, 32(4):315–323
32. Denhardt DT, Guo X: Osteopontin: a protein with diverse functions. FASEB J 1993, 7:1475–1482
33. Hwang SM, Lopez CA, Heck DE, Gardner CR, Laskin DL, Laskin JD, Denhardt DT: Osteopontin inhibits induction of nitric oxide synthase activity by inflammatory mediators in mouse kidney epithelial cells. J Biol Chem 1994, 269:711–715
34. Senger DR, Perruzzi CA, Gracey CF, Papadopoulos A, Tenen DG: Secreted phosphoproteins associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. Cancer Res 1988, 48:5770–5774
35. Senger DR, Perruzzi CA, Papadopoulos A: Elevated expression of secreted phosphoprotein I (osteopontin: 2AR) as a consequence of neoplastic transformation. Anticancer Res 1989, 9:1291–1300
36. Gardner HAR, Berse B, Senger DR: Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat1 fibroblasts. Oncogene 1994, 9:2321–2326
37. Giachelli CM, Bae N, Lombardi D, Majesky M, Schwartz S: Molecular cloning and characterization of 2B7, a rat mRNA which distinguishes smooth muscle cell phenotypes *in vitro* and is identical to osteopontin (secreted phosphoprotein I:2AR). Biochem Biophys Res Commun 1991, 177:867–873
38. Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM: Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest 1993, 92:1686–1696
39. Gadeau AP, Campan M, Millet D, Candresse T, Desgranges C: Osteopontin overexpression is associated with arterial smooth muscle cell proliferation *in vitro*. Arterioscler Thromb 1992, 13:120–125
40. Wax SD, Rosenfield CL, Taubman MB: Identification of a novel growth factor-responsive gene in vascular smooth muscle cells. J Biol Chem 1994, 269:13041–13047
41. Green RS, Lieb MB, Weintraub AS, Gacheru SN, Rosenfield CL, Shah S, Kagan HM, Taubman MB: Identification of lysyl oxidase and other PDGF-inducible genes in vascular smooth muscle cells by differential screening. Lab Invest 1995, 73:476–482
42. Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM: Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells *in vitro*. Circ Res 1994, 74:214–224
43. Taubman MB, Marmur JD, Rosenfield CL, Guha A, Nichtberger S, Nemerson Y: Agonist-mediated tissue factor expression in cultured vascular smooth muscle cells: role of calcium mobilization and protein kinase C activation. J Clin Invest 1993, 91:547–552
44. Cacace AM, Guadagno SN, Krauss RS, Fabbro D, Weinstein IB: The epsilon isoform of protein kinase C is an oncogene when overexpressed in rat fibroblasts. Oncogene 1993, 8:2095–2104
45. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, ed 2. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989
46. Brand SJ, Stone D: Reciprocal regulation of antral gastrin and somatostatin gene expression by omeprazole-induced achlorhydria. J Clin Invest 1988, 82(3):1059–1066
47. Irving JA, Lala PK: Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-β, IGF-II, and IGFBP-1. Exp Cell Res 1995, 217:419–427
48. Mann R, Mulligan RC, Baltimore D: Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 1983, 33:153–159
49. Bernanke DH, Markwald RR: Effects of two glycosaminoglycans on seeding of cardiac tissue cells into a collagen-lattice culture system. Anat Rec 1984, 210: 25–31
50. Turley EA, Erickson CA, Tucker R: The retention and ultrastructural appearances of various extracellular matrix molecules incorporated into three-dimensional hydrated collagen lattices. Dev Biol 1985, 109:347–369
51. Chambers AF, Hota C, Prince CW: Adhesion of metastatic *ras*-transformed NIH 3T3 cells to osteopontin, fibronectin, and laminin. Cancer Res 1993, 53:701–706
52. Butler WT: The nature and significance of osteopontin. Connective Tissue Res 1989, 23:123–136
53. Kleinman HK, McGoodwin EB, Martin GR, Klebe RJ: Localization of the binding site for cell attachment in the α1 (1) chain of collagen. J Biol Chem 1978, 256: 5642–5646
54. Meredith JE, Fazeli B, Schwartz MA: The extracellular matrix as a cell survival factor. Mol Biol Cell 1993, 4:953–961