Osteopontin Promotes Vascular Cell Adhesion and Spreading and Is Chemotactic for Smooth Muscle Cells In Vitro

Lucy Liaw, Manuela Almeida, Charles E. Hart, Stephen M. Schwartz, Cecilia M. Giachelli

Abstract

Osteopontin is an Arg-Gly-Asp-containing acidic phosphoprotein recently shown to be upregulated in vascular smooth muscle during rat arterial neointima formation and in human atherosclerotic plaques. Functional studies showed that osteopontin promoted adhesion of both cultured aortic endothelial cells and aortic smooth muscle cells. Adhesion of vascular cells to osteopontin was dose dependent and maximal when solutions containing 7 and 30 nmol/L osteopontin were used to coat wells for endothelial and smooth muscle cells, respectively. Smooth muscle cells adherent to osteopontin were spread after 60 minutes, whereas endothelial cells remained round, although flattened, at this time point but were spread at 90 minutes. Cell spreading on osteopontin was accompanied by the formation of focal adhesion plaques. A newly developed anti-osteopontin antibody completely inhibited adhesion of both cell types to osteopontin but not to fibronectin or vitronectin. In addition, the peptide GRGDSP blocked adhesion to osteopontin, suggesting that integrins mediate Arg-Gly-Asp-dependent adhesion. Indeed, an antibody against the \( \alpha_\beta_3 \) integrin neutralized adhesion of both endothelium and smooth muscle cells to osteopontin by \( \approx 50\% \), demonstrating that \( \alpha_\beta_3 \) is one osteopontin receptor on vascular cells. Osteopontin also promoted the migration of smooth muscle cells in a Boyden-type chamber, with half-maximal effects observed at 77 nmol/L osteopontin. Checkerboard analysis demonstrated that this stimulus was chemotactic in nature. Our findings suggest that osteopontin may be functionally important as an adhesive and chemotactic molecule for vascular cells, particularly when levels of osteopontin are dramatically increased, as is the case after arterial angioplasty and in atherosclerotic plaques. (Circ Res. 1994;74:214-224.)

Key Words • osteopontin • adhesion • migration
• smooth muscle • endothelium

Morphogenic processes are thought to contribute significantly to vascular pathologies such as restenosis and atherosclerosis, as well as to the normal ontogenic development of the vasculature. It is clear from studies of both endothelial cells and smooth muscle cells (SMCs) that proteins with cell adhesive properties may play key roles in mediating these events. Several examples are the growth effects of thrombospondin on both SMCs and endothelium, the contribution of laminin to in vitro angiogenesis, phenotypic changes in vascular SMCs plated on laminin or fibronectin substrates, and stimulation of endothelial cell migration with fibronectin. Thus, the ability of a molecule to provide an adhesive substrate for vascular cells may suggest a broad range of functions pertaining to cellular remodeling.

In a previous study, we discovered the expression of osteopontin, a secreted adhesive glycoprotein, in vascular SMCs by use of a differential cloning strategy aimed at identifying genes that would distinguish the phenotypically distinct SMC types we have observed in vitro. Subsequently, we showed that endothelial denudation of either the rat aorta or carotid artery caused a dramatic increase in osteopontin mRNA and protein synthesis selectively in SMCs forming the arterial neointima. The spatial and temporal pattern of osteopontin expression coincided with alterations in SMC phenotype, invasion of the intima, and proliferation, thus implicating a role for osteopontin in the early events leading to vascular remodeling and neointima formation in this model. The potential relevance of osteopontin expression in the progression of human vascular disease was further suggested by the focal expression of osteopontin in atherosclerotic plaques but not normal arteries.

Although osteopontin was originally identified as a matrix protein in bone, the protein has subsequently been found in kidney, inner ear, brain, placenta, decidua, bone marrow, a variety of epithelial cell surfaces, and tumor cells. The roles of osteopontin in these various tissues have not been clearly elucidated, although adhesion is one postulated function, since osteopontin contains a highly conserved attachment motif Arg-Gly-Asp (RGD) similar to that found in other adhesive matrix molecules that interact with cellular integrins. This idea has been supported by in vitro studies of bone, ligament, and transformed cells, where RGD-dependent attachment and spreading are promoted by osteopontin. Furthermore, osteopontin expression correlates with the metastatic potential of tumor cells, implicating osteopontin in processes controlling cellular invasiveness. In the present study, we have investigated whether osteopontin can provide an adhesive substrate for SMCs and endothelial cells, two cell types important in vascular remodeling processes. Additionally, we have tested the effects of osteopontin on the migration of smooth muscle cells, since this is a commonly proposed function for osteopontin that, nev-
N-Terminal Sequencing

N-terminal sequencing was carried out by the University of Washington Molecular Pharmacology Facility, Seattle, using an Applied Biosystems model 477A liquid-phase protein sequencer with an on-line model 120A phenylthiodyantoin analyzer.

Osteopontin Antibody

A goat was immunized with 50 μg rat osteopontin protein with Freund’s complete adjuvant to produce antibody OP199. Osteopontin was purified as described above but with an additional reverse-phase purification step involving high-pressure liquid chromatography over a VyDAC C4 column. This preparation was judged to be highly purified (>99% pure) on the basis of Coomassie and silver staining as well as N-terminal sequence analysis. Three booster injections of 50 μg each in Freund’s incomplete adjuvant were administered 3 weeks apart after the initial immunization. The first test bleeds were obtained 6 weeks after the initial injection and then after the following 3 weeks. Serum was purified over protein G capsules (Amicon, Beverly, Mass) to collect an IgG fraction. This fraction was diazylated, sterile-filtered, and stored frozen. Specificity was tested by enzyme-linked immunosorbent assay, Western blot analysis, and inhibition studies in functional assays and for cross recognition of vitronectin and fibronectin.

Western Blot Analysis

Conditioned medium was collected from confluent cultures of neonatal (pup) rat SMCs in serum-free medium and concentrated by use of Centricon-30 columns, and 1/250 of the total fraction was used in the blots probed with antibody OP199. Samples were boiled for 2 minutes, diluted 1:1 with sample buffer containing 100 mmol/L Tris (pH 6.8), 10% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol, and separated on a 10% SDS–polyacrylamide gel under reducing conditions. The proteins were blotted onto Zeta Probe membranes (Bio-Rad Laboratories, Richmond, Calif) overnight and incubated at room temperature as follows. Nonspecific binding was blocked by 10% nonfat dry milk with 2% normal rabbit serum for 1 hour, followed by either anti-osteopontin (1:1000 dilution for OP199) or normal goat IgG (at a dilution required to obtain the same concentration as OP199) with 1% normal rabbit serum for 2 hours. Blots were incubated for 1 hour with 1:400 dilution of biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, Calif). Peroxidase labeling was carried out with an avidin-peroxidase complex (ABC Elite, Vector Laboratories) as per the manufacturer’s instructions, using 4-chloro-1-naphthol as a substrate. Prestained molecular weight standards were used to estimate protein size (Bio-Rad Laboratories).

Adhesion Assay

Test adhesive substrates were diluted in PBS to stated concentrations, and 50 μL per well was added to 96-well Maxisorp plates (Nunc, Naperville, Ill) and placed overnight at 4°C. U937, a radiodination of osteopontin, vitronectin, and fibronectin, we have determined the amounts of each protein bound to the wells under these conditions (see the Table). After the coating period, wells were rinsed with PBS, and nonspecific binding sites were blocked with 10 mg/mL bovine serum albumin (BSA) at 37°C for 1 hour. Cells were detached by minimal trypsinization (1 to 2 minutes), placed immediately
Adhesion Assay

<table>
<thead>
<tr>
<th>Protein Bound, ng per well</th>
<th>Average coating efficiency, %</th>
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<tr>
<td>50 nmol/L*</td>
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<tr>
<td>100 nmol/L*</td>
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<tr>
<td>Osteopontin</td>
<td></td>
</tr>
<tr>
<td>66.8</td>
<td>114</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>48.1</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>173</td>
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</tbody>
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*Concentration of coating solution.

into an equal volume of 0.5 mg/mL soybean trypsin inhibitor (STI, Sigma), and centrifuged. The cell pellet was washed with STI and resuspended by centrifugation. Cells were resuspended in Waymouth's complete medium with 1 mg/mL BSA. Approximately 30,000 cells were placed in each well and allowed to adhere at 37°C for 30 to 90 minutes. In experiments in which RGD peptides or antibodies were used, they were added to wells immediately before cells. In studies with OP199, the antibody was allowed to interact with protein-coated wells for 30 minutes before the addition of cells. Nonadherent cells were rinsed off with PBS, and the remaining cells were fixed with 4% paraformaldehyde for 5 minutes. Cells were stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 minutes and rinsed in water. Cells were solubilized with the addition of 100 µL of 1% SDS and quantified in a microtiter plate reader at 595 nm. For direct quantification of adherent cells, plates were rinsed, trypsinized, and counted in a Coulter counter. Experiments described were performed in quadruplicate and repeated a minimum of three times. Mean±SEM data from representative experiments are shown.

Immunocytochemistry

For actin staining, cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes, permeabilized with acetone for 3 minutes, and stained with 2 U of Bodipy 505/512 phallacidin (Molecular Probes, Inc, Eugene, Ore). For vinculin staining, cells were fixed in 4% paraformaldehyde at room temperature for 15 minutes and permeabilized for 2 minutes with 0.2% Triton X-100 in 50 mmol/L Tris (pH 7.5). Monoclonal anti-vinculin clone (VIN-11-5, Sigma) was used at a 1:60 dilution and incubated for 1 hour, followed by fluorescein-conjugated rabbit immunoglobulin to mouse immunoglobulins (DAKO, Carpinteria, Calif) in a 1-hour incubation. All slides were mounted with Vectashield mounting media (Vector Laboratories).

Migration Assay

Test substances were diluted to appropriate concentrations in Waymouth's complete medium containing 200 µg/mL BSA and placed in the bottom wells of a modified Boyden chemotaxis chamber (Neuro Probe Inc, Cabin John, Md), unless otherwise indicated. A polycarbonate filter with 8-µm pores was placed between the test proteins and the upper chamber. Identical results were obtained when filters precoated with collagen type I (Vitrogen, Celtrix Laboratories, Palo Alto, Calif) were used. Freshly trypsinized rat aortic SMCs were washed twice and resuspended in Waymouth's media containing 200 µg/mL BSA. Fifty thousand cells were placed in the top wells and allowed to migrate for 4 to 6 hours at 37°C in a humidified chamber. After the incubation period, cells that had migrated to the bottom of the filter were fixed with methanol and stained with hematoxylin. Migration was quantified by cell counts of three random ×400 high-power fields in each well. Each experiment was performed in quadruplicate and repeated at least twice.

Results

Purification of Osteopontin

Osteopontin was purified from the conditioned medium of pup rat SMC cultures. N-terminal sequencing of this preparation detected a sequence that was identical to that of osteopontin purified from rat bone (Fig 1). This preparation was further analyzed by gel electrophoresis and Western blot analysis with two different antibodies made against rat osteopontin. Fig 2a shows a Coomassie-stained gel detecting a doublet at ≈64 and 66 kD. These bands represent osteopontin as demonstrated by Western blot analysis (Fig 2b) with MPIIIIB10(1), a monoclonal antibody against rat osteopontin. This antibody also detected a small amount of an ≈55-kD protein and several higher

![Image](http://circres.ahajournals.org/)

**Fig 1.** Comparison of osteopontin N-terminal sequences. Osteopontin purified from the conditioned medium of rat smooth muscle cell (SMC) cultures had an identical deduced and actual N-terminal sequence as osteopontin purified from rat bone.8,16 The deduced N-terminal sequences of human,17,18 bovine,19 porcine,20 and murine (deduced and actual are identical)21,22 osteopontin are shown. The determined N-terminal sequence of osteopontin from human bone differs from the predicted sequence in the underlined residues,23 where a glutamic acid replaces the serine and a glutamine replaces the glutamic acid.
Fig 3. Graphs showing quantification of adhesion assays with cell counts vs cell staining. Wells were coated with fibronectin solutions ranging in concentration from 0.45 to 113 nmol/L. Bovine aortic endothelial cells were allowed to adhere in wells for 60 minutes. Parallel groups of quadruplicates were quantified one of two ways: (1) adherent cells were fixed, stained with 0.5% toluidine blue, washed, and solubilized with 1% sodium dodecyl sulfate, and absorbance was read at 595 nm in a microtiter plate reader (a); and (2) plates were rinsed, and adherent cells were trypsinized with 0.05% trypsin and counted in a Coulter counter (b). Values represent means and standard error. Mean absorbance was plotted against total cell number from the same experiment, and a simple curve shows a close to linear relation between the two methods of quantification (c).

Adhesion of Vascular Endothelial Cells to Osteopontin

BAECs adhered to wells coated with substrates of osteopontin, fibronectin, and vitronectin in a dose-dependent manner (Fig 4a). Fibronectin and vitronectin were used as adherent substrates on the basis of previous reports of their ability to promote endothelial cell adhesion. Dose-dependent adhesion was observed when solutions containing up to 100 nmol/L osteopontin were used to coat microtiter wells. Half-maximal effects were observed using coating solutions containing 7 nmol/L osteopontin (0.45 μg/mL), 11 nmol/L fibronectin (2.5 μg/mL), and 7 nmol/L vitronectin (0.49 μg/mL). The adherent cells were firmly attached but appeared rounded at 30 to 60 minutes of incubation and began to spread at 75 minutes. BAECs were highly spread after 90 minutes of incubation and formed a monolayer at 120 minutes (Fig 4b). The time course of adhesion and spreading on osteopontin was identical to that of cells plated on either fibronectin or vitronectin substrates (not shown). Cells plated in control wells with only BSA present did not adhere and were washed off during the rinses.

Active protein synthesis was not necessary for the adhesion of BAECs to osteopontin (Fig 4b, photomicrograph d), since pretreatment of cultures with 25 μg/mL cycloheximide for 2 hours and maintenance of the drug during the assay period did not diminish adhesion of the cells. Maximal adhesion to osteopontin was obtained at 90 minutes, when 80% of BAECs were adherent and 76% of cycloheximide-treated BAECs had adhered to the substrate. These results indicate that ongoing synthesis of other proteins is not responsible for BAEC adhesion to an osteopontin substrate. In addition, since interactions of cells with matrix proteins often leads to organization of the cytoskeleton, we tested whether such an organization would occur in cells plated on osteopontin. Cells adherent to osteopontin...
were fixed and stained with either fluorescein-conjugated phallicidin (binds only to filamentous actin; Fig 4b, photomicrograph c) or a monoclonal antibody to vinculin (Fig 4b, photomicrograph f). At 60 minutes, BAECs were still rounded on an osteopontin substrate, and the actin staining was faint and perinuclear with the absence of filamentous organization. BAECs adherent to osteopontin and allowed to spread organized their actin filaments and formed focal contacts, as demonstrated by the localization of vinculin to discrete contact areas within the cell. These findings were similar to cells that had been pretreated with cycloheximide before and during experiments.

**Adhesion of Vascular SMCs to Osteopontin**

Adult rat aortic SMCs were also tested for their ability to adhere to substrates of osteopontin compared with other extracellular matrix proteins. Fig 5a shows that SMCs adhered in a dose-dependent manner to wells previously coated with substrates of osteopontin, fibronectin, and vitronectin, with maximal adhesion between 100 and 200 nmol/L of each substrate. Half-maximal responses were observed when solutions of 30 nmol/L osteopontin (3.9 µg/mL), 9 nmol/L vitronectin (0.63 µg/mL), and 11 nmol/L fibronectin (2.4 µg/mL) were used for coating. The SMCs were adherent at 30 minutes and had a spread phenotype (Fig 5b) on all of the substrates at 60 minutes into the assay. No cellular adhesion was observed in control wells coated with only BSA.

Pretreatment of cultures and maintenance of cycloheximide in the assay did not significantly decrease adhesion or spreading of SMCs (Fig 5b, photomicrograph d). The number of SMCs adherent to osteopontin was lower than that to substrates of fibronectin or vitronectin at 60 minutes, indicating either that there are fewer osteopontin receptors on these cells or that complete binding equilibrium did not occur at 60 minutes. In fact, we found that the maximal number of adherent cells occurred at 2 hours and was 80% of the total input of SMCs and 73% of the total in cycloheximide-treated cultures. It is also a possibility that not all of the SMCs in our cultures contain identical receptor profiles. Cytoskeletal organization was also assessed after SMC adhesion to osteopontin by immunocytochemistry using fluorescein-conjugated phallicidin (interacts only with filamentous actin) or an anti-vinculin antibody. At early times, such as 30 minutes into the assay when SMCs were not well spread on osteopontin, the actin staining showed faint perinuclear localization and an absence of filaments. Fig 5b (photomicrographs e and f) shows that, in SMCs spread on an osteopontin substrate, actin filaments were detectable and vinculin was localized to punctate areas near the cell periphery. The same results were observed with SMCs that had been treated with cycloheximide.
Antagonists of Endothelial and SMC Adhesion to Osteopontin

The antibody to osteopontin, OP199, was tested for its ability to block endothelial and SMC adhesion to substrates of osteopontin, vitronectin, and fibronectin. Fig 6a shows that increasing concentrations of OP199 inhibited BAEC adhesion to osteopontin, without affecting adhesion to vitronectin or fibronectin substrates. A concentration of 50 μg/mL OP199 inhibited 20% BAEC adhesion to osteopontin, 100 μg/mL inhibited 56% adhesion, and 200 μg/mL inhibited 95% adhesion. This was in contrast to the effects of 200 μg/mL normal goat IgG, which did not affect BAEC adhesion to osteopontin. Adhesion to substrates of vitronectin and fibronectin were not inhibited at any dose of OP199 antibody. Antibody OP199 strongly inhibited interaction of rat SMC with osteopontin substrates (Fig 6b). A concentration of 50 μg/mL inhibited 56% adhesion, and 100 μg/mL inhibited 96% SMC adhesion to osteopontin. These effects were specific, since OP199 did not reduce SMC adhesion to substrates of vitronectin or fibronectin and 100 μg/mL normal goat IgG had no effect.

Synthetic hexamer peptides corresponding to the sequences GRGDSP and GRGESP were used to test the importance of the osteopontin RGD sequence in mediating cell attachment. Fig 7a shows that BAEC adhesion to osteopontin was inhibited with 100 μmol/L RGD hexamer peptide with significant inhibition seen with 10 μmol/L peptide, very similar to GRGDSP competition seen on fibronectin and vitronectin substrates (data not shown). The RGD peptide was also able to inhibit SMC adhesion to osteopontin at a concentration of 100 μmol/L (Fig 7b). Similar inhibition was seen with SMC adhesion to fibronectin and vitronectin (data not shown). The concentrations of GRGDSP used to inhibit adhesion are typical of studies in which RGD-containing peptides are used to compete for RGD-dependent integrin binding. In contrast, even very high concentrations (≥500 μmol/L) of the GRGESP peptide showed only partial inhibitory activity in either cell type.

These results suggested that the cellular receptor for osteopontin on vascular cells might be an integrin molecule, and other researchers have provided evidence that the αβ, integrin is an osteopontin receptor. Therefore, we used a neutralizing monoclonal antibody against αβ, LM609, to test whether this receptor mediated osteopontin interaction with endothelium and SMCs. Fig 8a shows that LM609 blocked 45% BAEC adhesion to osteopontin. Since LM609 does not functionally neutralize rat αβ, we tested a human SMC line that adhered to osteopontin in a manner similar to that found with rat SMCs. LM609 was also able to partially block the adhesion of human SMCs to osteopontin (Fig 8b), and this interaction was completely blocked by OP199. As expected, adhesion of these cells to vitronectin was inhibited by LM609 and not affected by OP199. Inhibition of both cell types to osteopontin was only partially neutralized by
LM609 even at concentrations of 100 μg/mL purified LM609 IgG (not shown). The result of LM609 only partially inhibiting adhesion to a vitronectin substrate is consistent with the probable presence of multiple vitronectin receptors, including α3β1 and αvβ3.

The interaction of BAECs with osteopontin also led to clustering of α3β1 receptors, demonstrated by immunocytochemical localization with LM609 (Fig 8c). One hour after adhesion to osteopontin, α3β1 was distributed in a punctate pattern near the periphery of BAEC cell borders, indicating formation of focal contacts and cellular organization on an osteopontin substrate. The pattern of α3β1 localization was similar to that found in areas enriched with vinculin. Cells were also stained with normal mouse IgG as a control or without a primary antibody, and no specific immunofluorescence was noted (not shown).

**Osteopontin Effects on SMC Migration**

We functionally characterized osteopontin further by testing for effects on SMC migration using a Boyden-type chamber assay. Osteopontin stimulated the dose-dependent migration of rat vascular SMCs as shown in Fig 9a. Half-maximal effects were observed with 77 nmol/L osteopontin. To determine whether the migratory stimulus was chemotactic or chemokinetic in nature, the location of osteopontin in the migration chamber was varied. A chemotactic effect was noted, since a gradient of osteopontin (Fig 9b, bottom only) was required for maximal migratory effect. Vitronectin was
also chemotactic for rat SMCs in this assay, in agreement with previous results. The specificity of the migratory effect was determined by inhibition with anti-osteopontin antibody, OP199, but not with normal goat IgG (Fig 9c). OP199 was not a general inhibitor of migration, since it had no effect on the migration of rat vascular SMCs to the potent chemotactic agent, platelet-derived growth factor (data not shown).

**Discussion**

We report that purified smooth muscle–derived osteopontin was able to promote adhesion of both vascular SMCs and endothelial cells in culture via an RGD-mediated interaction with at least one integrin, αβ3. Furthermore, osteopontin was shown to be chemotactic for SMCs. In addition, we describe the preparation and characterization of an antibody against osteopontin, OP199, which effectively and specifically neutralized the adhesive and migratory effects of osteopontin on these cell types. Our studies are the first to demonstrate that osteopontin is an adhesion molecule for vascular endothelium and SMCs and a chemotactic factor for vascular SMCs. Our findings suggest that osteopontin may share some functions with other proteins, such as collagen types I and III, laminin, fibronectin, and vitronectin in endothelial cells and in collagen types I and IV in smooth muscle, all known to function as adhesive substrates and, in some cases, migratory stimuli for vascular cells.

Our studies are in general agreement with previous work characterizing osteopontin as an RGD-dependent adhesive molecule for a variety of cell types. Most recently, Brown et al. showed that osteopontin could facilitate adhesion of human intestinal SMCs in an RGD-dependent manner, but the receptor responsible for this interaction was not determined. In vascular SMCs and endothelial cells, as well as osteoclasts, the integrin αβ3 appears to be one receptor for osteopontin binding (the present study and Reference 31). However, in our experiments, an anti-αβ3 antibody, LM609, only partially inhibited SMC and BAEC binding to osteopontin, whereas an RGD-containing hexamer completely abolished the adhesive interaction. These data suggest that other integrin-like molecules might also be important for binding of vascular cells to osteopontin. Clearly, determining whether other integrins are involved in osteopontin binding to vascular cells as well as other cell types is a high priority and might shed some light on the roles of osteopontin in diverse tissues.

Whereas a migratory potential has been widely postulated for osteopontin on the basis of its overexpression in metastatic cells, its ability to stimulate macrophage accumulation after peritoneal injection in mice, and its presence in actively remodeling bone, no data directly supporting this idea have been previously reported. Our studies using the modified Boyden chemotaxis chamber show that purified osteopontin could stimulate smooth muscle migration in a dose-dependent manner, with half-maximal effects observed at 77 nmol/L osteopontin. In contrast, using a similar chemotaxis system, migration of gingival fibroblasts to osteopontin was not observed at concentrations as high as 20 µg/mL. This discrepancy no doubt reflects differences in the effects of osteopontin on the different cell types used in each study, since gingival fibroblasts, like vascular smooth muscle, showed enhanced attachment to osteopontin. The differences in function of osteopontin in various cell types might depend on the complement of different receptors or signaling pathways prevalent in a given cell type.

The adhesive and migratory properties of osteopontin in vascular cells observed in the present study suggest a potential role for this molecule in the morphogenic events seen during vascular development or repair. After balloon angioplasty, for example, SMCs undergo phenotypic, proliferative, and migratory changes resulting in the formation of a smooth muscle–rich neointimal lesion similar to that seen in many vascular pathologies in humans. Indeed, we recently showed that osteopontin protein and mRNA levels were dramatically elevated after arterial balloon angioplasty in rat carotid arteries and aortas and...
in the human atherosclerotic plaque. In contrast, normal adult arteries contained very little osteopontin. Furthermore, basic fibroblast growth factor, transforming growth factor-\(\beta\), and angiotensin II, all implicated in the arterial injury response, induced osteopontin mRNA and protein levels in cultured vascular SMCs.\(^6\) It is interesting to note that other molecules appearing in the vascular cell microenvironment after injury, ie, fibrinogen, von Willebrand factor, fibronectin, elastin, and thrombospondin, also promote adhesion and organization of vascular cells.\(^2,27\) Many of these adhesive proteins interact with cells via integrin receptors, which can mediate adhesion and migration of both SMCs and endothelial cells. A second category of proteins expressed after arterial injury includes those that are antiadhesive or mediate cell detachment from matrix factors. Examples of these proteins are tenasin,\(^43\) generally considered an antiadhesive molecule, and a number of proteases hypothesized to facilitate cell migration by releasing cells from their matrixes.\(^44\) The de novo production of an adhesive protein substrate may be a prerequisite for cellular responses to injury, and it is likely that a balance of adhesion and deadhesion are necessary for processes such as invasion and migration.

Our findings suggest the following hypothetical model for osteopontin regulation and function during arterial neointimal formation: Endothelial injury stimulates the release of vasoactive factors (basic fibroblast growth factor, transforming growth factor-\(\beta\), platelet-derived growth factor, angiotensin II, and no doubt others) from platelets and endothelial and medial SMCs, initiating

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**Fig 9.** Graphs showing chemotactic effects of osteopontin (OP) on smooth muscle cells. a, Dose-response curve is shown for rat aortic smooth muscle cell migration to smooth muscle–derived OP. Cells migrated per \(\times400\) high power field (cells/HPF) are given as mean±SD for a representative experiment performed in quadruplicate. b, Checkerboard analysis of smooth muscle cell migration to OP is shown. Ten micrograms per milliliter OP (154 nmol/L) or 4 \(\mu\)g/mL vitronectin (VN, 57 nmol/L) were either omitted (neither) or added to the top, bottom, or top and bottom Boyden chamber compartments, and smooth muscle cells were allowed to migrate for 6 hours. Mean±SD values were determined as in a. c, Migration of smooth muscle cells to 7.5 \(\mu\)g/mL OP (115 nmol/L) was assessed in the presence of increasing concentrations of antiosteopontin antibody, OP199, or normal goat IgG. Results are given as mean±SD cells/HPF for a representative experiment performed in quadruplicate.
phenotypic changes, proliferation, and migration in medial SMCs. Some of these molecules, in addition, upregulate osteopontin expression. Osteopontin then works in combination with proteases, integrins, and other matrix molecules to promote adhesion and facilitate movement of SMCs into the intimal compartment. In this scenario, osteopontin would simultaneously favor the reendothelialization of the wound by acting as an adhesive substrate (and possibly migratory stimulus?) for endothelial cells. Effects of osteopontin on SMCs and endothelial cells would thus be synergistic in promoting arterial repair. Other hypotheses also exist, none of which are mutually exclusive. For example, osteopontin may be critical for maintenance of the distinct phenotypes of SMCs seen in vascular lesions, as suggested by its differential expression in phenotypically distinct cultured SMCs.5,45 Alternatively, osteopontin may be involved in some way in cell cycle progression, as suggested by the studies of Gadeau et al.46 Experiments using the specific neutralizing antibody against osteopontin (OP199) will allow us to directly test these various hypotheses in vivo in the rat balloon injury model and are currently in progress.

Acknowledgments

This study was supported by National Institutes of Health (NIH) grants HL-18654 and HL-03174. Dr Giachelli is also supported by grant 3124R1 from the Council for Tobacco Research, and L. Liaw is supported by NIH training grant HL-07312. Special thanks are extended to William Downey (Zymogenetics) and Francine Stanton for their expert technical assistance in protein purification and migration assays, respectively.

References

5. Giachelli C, Bae N, Lombardi D, Majesky M, Schwartz S. Molecular cloning and characterization of 27B, a rat mRNA which distinguishes smooth muscle cell phenotypes in vitro and is identical to osteopontin (secreted phosphoprotein 1, 2aB). Biochem Biophys Res Commun. 1991;177:867-873.


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Circ Res. 1994;74:214-224
doi: 10.1161/01.RES.74.2.214

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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