SPARC (BM-40, Osteonectin) Inhibits the Mitogenic Effect of Vascular Endothelial Growth Factor on Microvascular Endothelial Cells*

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SPARC (secreted protein, acidic and rich in cysteine) is a matricellular protein that modulates cell adhesion and proliferation and is thought to function in tissue remodeling and angiogenesis. In this study, we demonstrate that SPARC inhibits DNA synthesis by >90% in human microvascular endothelial cells (HMEC) stimulated by the endothelial cell mitogen vascular endothelial growth factor (VEGF). Peptides derived from SPARC domain IV, which contains a disulfide-bonded EF-hand sequence and binds to endothelial cells, mimicked the effect of native SPARC. The inhibition was also observed with a peptide from the follistatin-like domain II, whereas peptides from SPARC domains I and III had no effect on VEGF-stimulated DNA synthesis. The inhibition of HMEC proliferation was mediated in part by the binding of VEGF to SPARC. The binding of 125I-VEGF to HMEC was reduced by SPARC and SPARC peptides from domain IV in a concentration-dependent manner. In a radioimmune precipitation assay, peptides from SPARC domains II and IV each competed with native SPARC for its binding to VEGF. It has been reported that VEGF stimulates the tyrosine phosphorylation and activation of mitogen-activated protein kinases Erk1 and Erk2. We now show that SPARC reduces this phosphorylation in VEGF-stimulated HMEC to levels of unstimulated controls. SPARC thus modulates the mitogenic effect of VEGF through an interaction with PDGF-AB and -BB and a subsequent inhibition of binding to cognate cell-surface receptors (6). SPARC also reduces the proliferative and migratory effects of bFGF on endothelial cells. A direct interaction between bFGF and SPARC, however, has not been established (7).

VEGF is an endothelial cell mitogen that also increases vascular permeability (8). There are five different human VEGF isoforms, consisting of monomers of 121, 145, 165, 189, and 206 amino acids, that are derived from a single gene by alternative splicing (9–11). Each monomer is capable of forming an active homodimer that is secreted either exclusively into the culture medium (VEGF121), localized at the cell surface, and/or associated with the ECM, possibly as a result of differential binding to heparan sulfate proteoglycans. VEGF exhibits a 20% similarity in amino acid sequence to PDGF A and B chains and a 53% similarity to placenta growth factor (12). The class III receptor tyrosine kinases Flt-1 and KDR/Flk-1 interact specifically with VEGF (13). An essential role for Flk-1 in embryonic vasculogenesis has been established, since Flk-1−/− mice exhibited significantly reduced formation of blood islands and died in utero. In contrast, mice lacking the Flt-1 receptor did form blood islands, but the morphogenesis of the vasculature was impaired (14, 15). A major stimulus for VEGF/VEGF receptor expression in adult tissue is hypoxia, a condition resulting from ischemia (16, 17). Recent studies have also shown that VEGF could regulate angiogenesis-dependent tumor growth (18), in part through induction of integrin receptors that recognize one or more collagen types.

We have proposed that SPARC modulates, directly or indirectly, the effects of growth factors and ECM on cell behavior (3). In this study, we demonstrate that SPARC interferes with VEGF-stimulated endothelial cell proliferation. Since pericytes and endothelial cells respond to PDGF and VEGF, respectively, SPARC appears to be a significant factor in the regulation of vascular growth.
**EXPERIMENTAL PROCEDURES**

**SPARC Protein and Synthetic Peptides—** SPARC was purified from the culture medium of murine parietal yolk sac carcinoma cells (19); SPARC peptides were synthesized and purified by high performance liquid chromatography (20). Concentrations in phosphate-buffered saline (PBS), pH 7.4, were determined by absorbance at 280 nm (extinction coefficient for a 1 nm solution at 280 nm is 0.92 for SPARC and 7.21 for peptides 4.2 and 4.0). Concentrations for peptides 2.1 and 3.4 were calculated by weight only.

**Cell Culture and [³H]Thymidine Incorporation—** Low passage (5–10) human dermal microvascular endothelial cells (HMEC) (Clonetics, San Diego, CA) were grown on 1% gelatin-coated flasks in fully supplemented MCDB 131 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Summit Biotechnologies, Ft. Collins, CO), 50 μg/ml endothelial cell growth supplement (Biomedical Technologies, Stoughton, MA), 20 μg/ml heparin, and 2 mM l-glutamine (Sigma).

Confluent HMEC were growth-artered by incubation in MCDB 131, devoid of serum and growth supplement, for 24 h. Cells were replated in fully supplemented MCDB 131 at a density of 25,000 cells/well in a 24-well plate. Within 2 h, 90% of the cells were attached. The medium was replaced with MCDB 131 containing heparin and glutamine only. SPARC, SPARC peptides and/or VEGF (PeproTech, Rocky Hill, NJ) was added for 20 h, and 1 μCi/ml (3.7 × 10⁸ Bq/ml) [³H]thymidine was present during the last 4 h. Material precipitated in trichloroacetic acid was hydrolyzed in 0.2 N NaOH and was subsequently dissolved in 3 ml of Ecolume (ICN, Costa Mesa, CA) for scintillation counting (5).

**Ligand Binding Assay and Radioimmunoassay—** VEGF and fibronectin were separated by SDS-PAGE, transferred to nitrocellulose, blocked with 5% milk/0.05% Tween 20 (Bio-Rad), and incubated for 3 h at room temperature with [¹²⁵I]-SPARC (1 pm), in the presence or absence of a 250-fold molar excess of unlabeled SPARC. Proteins that bound [¹²⁵I]-SPARC were visualized by autoradiography (6).

[¹²⁵I]-SPARC was incubated with VEGF as indicated and was immunoprecipitated with goat anti-human VEGF IgG (R&D Systems, Minneapolis, MN) and Protein G-Sepharose™ (Pharmacia, Piscataway, NJ) for 2 h at 4°C. The immunocomplex was precipitated for 2 h with Protein A-Sepharose™ beads washed with 50 mM Tris-HCl in 150 mM NaCl, boled in Laemmli buffer (21), and resolved by SDS-PAGE on 10% gels under nonreducing conditions (7).

**[¹²⁵I]-VEGF Binding to HMEC—** The binding of [¹²⁵I]-VEGF to cells was performed as described for [¹²⁵I]-PDGF binding (22), with minor variations. Briefly, cells were grown on noncoated plastic since it was found to decrease nonspecific binding without affecting the specific binding of [¹²⁵I]-VEGF. Confluent HMEC were incubated and growth supplement-free MCDB 131 for 24 h. Cells were rinsed once with ice-cold MCDB 131 containing 0.025 mM HEPES and 2.5 mM/ml bovine serum albumin (BSA). [¹²⁵I]-VEGF with or without SPARC was added at the indicated concentrations and was incubated at 4°C for 3 h on a platform shaker. The cells were washed three times with PBS containing 1 mg/ml BSA and 0.25 mM CaCl₂, and were incubated for 5 min in the presence of 0.5% Nonidet P-40, 150 mM NaCl, 150 mM HEPES, pH 7.4, 10% glycerol; Complete Protease-Inhibitor Mixture™ (Boehringer Mannheim). The insoluble material was removed by centrifugation for 20 min at 12,000 × g at 4°C, and the cell lysate was incubated overnight at 4°C with rabbit immune serum for 1 h. After three rinses in PBS, the sections were incubated with mouse anti-human phosphotyrosine IgG (Upstate Biotechnology, Lake Placid, NY). Immunoreactivity was detected by avidin–rhodamine (Vector Laboratories, Burlingame, CA). Immunoreactivity was detected by goat anti-rabbit IgG conjugated to horseradish peroxidase and visualized with 3,3′-diaminobenzidine as peroxidase substrate (Sigma).

**Immunohistochemistry—** Sagittal sections of mouse embryos (gestational day 12) were embedded in paraffin and were immersed in methyl Carnoy’s fixative (60% methanol, 30% chloroform, 10% acetic acid). Paraffin was removed by two 5-min washes each with xylene, ethanol, and PBS. Sections were incubated with 200-fold molar excess of unlabeled VEGF and/or 0.6 μM SPARC, the established ED₅₀ for inhibition of endothelial cell proliferation (5), in serum-free medium. VEGF stimulated an increase in [³H]thymidine incorporation of 80–100% whereas SPARC alone had no effect on DNA synthesis at a concentration of 0.6 μM (Fig. 1A). Therefore, the change in cell shape elicited by 0.6 μM SPARC did not result in a diminution of DNA synthesis in these cells. When SPARC and VEGF were added simultaneously to the cells, no significant increase in DNA synthesis compared with the solvent control was apparent (Fig. 1A). Moreover, simultaneous addition of SPARC and VEGF did not result in increased cell number, as measured by cell counts (data not shown). SPARC has four structurally different regions (domains I–IV) (25, 26); domains III and IV comprise an extracellular Ca²⁺-binding module (27). SPARC peptides 4.2 (amino acids 254–273) and 4.0 (amino acids 248–285) are located at the C terminus, within domain IV. Increasing concentrations of peptide 4.0 alone reduced [³H]thymidine incorporation by 82% (Fig. 1B), as noted for other endothelial cells (28). Addition of peptides 4.2 or 4.1 (amino acids 54–73) to HMEC stimulated by VEGF also inhibited cell proliferation (Fig. 1C). Peptide 3.4 (amino acids 184–303), however, had no significant effect on VEGF-stimulated DNA synthesis (Fig. 1C). These results demonstrate that SPARC and SPARC peptides from domains II and IV abrogated significantly the mitogenic effect of VEGF on HMEC. Similar levels of inhibition of VEGF-stimulated proliferation by SPARC were observed in human aortic endothelial cells and human iliac arterial endothelial cells (data not shown).

**VEGF Binds to ¹²⁵I-SPARC—** To understand how SPARC inhibits VEGF-stimulated endothelial cell proliferation, we incubated [¹²⁵I]-SPARC with VEGF (200 ng) and fibronectin (1 μg) that had been separated by SDS-PAGE and transferred to nitrocellulose (Fig. 2). [¹²⁵I]-SPARC bound to VEGF but not to...
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125I-VEGF. At a concentration of 0.4 nM, 125I-VEGF
was incubated with excess SPARC peptides, the amount of
binding was maximal; half-maximal binding occurred at a con-
centration of 48 pM. In the presence of 0.2 mM peptide 4.2, the
binding was reduced by 40%, with no appreciable change in affinity. In
contrast, low affinity binding was not affected significantly by
the addition of peptide 4.2.

The interaction of SPARC and SPARC peptides from
domains II and IV with VEGF is expected to result in reduced
splicing of HMEC—

SPARC Peptide 4.2 Inhibits the Binding of 125I-VEGF to
fibronectin. When the blot was incubated with 125I-SPARC in
the presence of a 250-fold molar excess of unlabeled
SPARC (±) inhibited the binding of 125I-SPARC to VEGF.

Table I

Binding of 125I-SPARC to VEGF is competed by
specific SPARC peptides

| SPARC peptide competitor | 125I-SPARC, % of total precipitate |
|--------------------------|----------------------------------|
| No peptide               | 100                              |
| Peptide 2.1              | 63 ± 2.06                       |
| Peptide 3.4              | 97 ± 6.4                         |
| Peptide 4.2ND*           | 54 ± 5.2                         |
| Peptide 4.2              | 15 ± 1.7                         |

* Peptide 4.2 in which Asp
was replaced by Asn, and Ala
was replaced by Asp.

Three independent experiments were quantified by densitometry. Numbers represent mean value ± S.D., normalized to the lane without peptide competitor.

SPARC Peptide 4.2 Inhibits the Binding of 125I-VEGF to
HMEC—The interaction of SPARC and SPARC peptides from
domains II and IV with VEGF is expected to result in reduced
levels of VEGF available for binding to its receptors. To test
this prediction, we incubated cells with increasing concen-
trations of 125I-VEGF. At a concentration of 0.4 nM, 125I-VEGF
binding was maximal; half-maximal binding occurred at a con-
centration of 48 pM. In the presence of 0.2 mM peptide 4.2, the
maximum binding of 125I-VEGF was reduced by 40% (Fig. 3A).
Increasing concentrations of peptide 4.2 added in the presence of
130 pM 125I-VEGF decreased the binding of VEGF to HMEC
by 70% of initial binding (Fig. 3B). Scatchard analysis indicated
two types of binding sites: a low affinity site (Kd = 4.2 × 10^{-10} M,
approximately 3.2 × 10^5 receptors/cell), and a high affinity site (Kd = 2.1 × 10^{-11} M, approximately 1.2 × 10^9 receptors/
cell) (Fig. 3C).

The addition of peptide 4.2 led to a reduction in the
number of high affinity receptors available for 125I-VEGF
binding by 40%, with no appreciable change in affinity. In
contrast, low affinity binding was not affected significantly by
the addition of peptide 4.2.
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**DISCUSSION**

In adult tissue, endothelial cells from the microvasculature participate in angiogenesis during wound healing, a process during which both SPARC and VEGF are expressed (31, 32). In this study, we present evidence that SPARC modulates the mitogenic activity of VEGF. Specifically, the mitogenic effect of VEGF on HMEC was abrogated in the presence of SPARC or peptides from different domains of SPARC. Previous work has established that SPARC counteracts the proliferative effect of PDGF-BB and -AB (6) on human fibroblasts by a direct binding to these mitogens. Moreover, both chemotaxis and proliferation mediated by bFGF in endothelial cells are inhibited by SPARC (7). Another matricellular protein, thrombospondin 1, reduced bFGF-induced endothelial cell proliferation within a concentration range similar to that of SPARC used in the experiments reported here (33). The concentrations of SPARC and SPARC peptides that produced 50–90% inhibition of proliferation (0.6 μM) for SPARC and 200 μM for the 20-amino acid SPARC peptides; Fig. 1) are completely consonant with previously pub-
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lished reports, in which SPARC was shown to modulate cell cycle progression (5), inhibit binding of PDGF-AB and -BB to its receptors (6), and regulate endothelial cell shape and barrier function (34). Disparities between concentrations of peptides and protein that are necessary to effect biological results in vitro are reported frequently and are most likely due to differences in the loss or destabilization of tertiary structure that is commonly seen in sequences of 20–40 amino acids (see Ref. 20, and references therein).

Previous reports have shown inhibition of the mitogenic activity of VEGF by anti-angiogenic factors such as the 16-kDa fragment of prolactin (29) and the chemokine platelet-factor-4 (PF-4) (35). PF-4 has been shown to inhibit the proliferation of endothelial cells cultured from macro- and microvessels within a molar concentration range similar to that of SPARC used in the experiments reported here (36, 37). PF-4 inhibits the binding of VEGF165 to its cell surface receptors on bovine aortic endothelial cells by interference with the binding of VEGF to heparan sulfates on the cell surface. That the cellular proliferation induced by nonheparin-binding VEGF121 was also decreased by PF-4 indicates an alternative mechanism as well. Recently, a peptide corresponding to the exon 7-encoded domain of VEGF165 was shown to inhibit the proliferation of human umbilical vein endothelial cells in response to the VEGF165 and VEGF121 isoforms (38).

Mechanisms of VEGF signal transduction through the receptor tyrosine kinases Flt-1 and KDR have not been resolved definitively. Different effects have in fact been attributed to the two VEGF receptors in porcine aortic endothelial cells that had been transfected with flt-1 or kdr (39). Flt-1 was shown to mediate membrane ruffling, whereas mitogenicity was induced after ligand binding to KDR (30, 39). Flt-1-expressing cells showed a ligand-dependent autophosphorylation of Flt-1, as well as phosphorylation of the GTPase-activating protein, phospholipase Cγ, and two members of the Src family of proteins (Fyn and Yes), but did not induce mitosis. In KDR-expressing cells, VEGF stimulated the phosphorylation of Shc, GTPase-activating protein, Grb2, and Nck (39). These adapter proteins are implicated in the mitogenic signaling of many tyrosine kinases that lead to the activation of the Ras-MAPK pathway (40). Human endothelial cells from microvessels have been reported to express both VEGF receptors (39), and our data confirm these observations. We suggest a direct interaction of SPARC with VEGF, and the subsequent inability of VEGF to bind to its receptors, as the predominant mechanism by which the proliferative activity of VEGF is inhibited. Tyrosine phosphorylation of Flt-1 upon VEGF binding did not take place in the presence of SPARC (Fig. 4). In our studies, SPARC inhibited the binding of VEGF to Flt-1 (Fig. 3) and its subsequent activation (Fig. 4) significantly more efficiently than it affected the binding of VEGF to KDR. Both receptors were nonetheless phosphorylated on tyrosine in the presence of VEGF alone (Fig. 4). This result indicates further alternative mechanisms by which SPARC inhibits the mitogenic activity of VEGF (see below).

In contrast to previous results in transfected porcine aortic endothelial cells (39), it is possible that the mitogenic signal in our cell system is transduced by Flt-1. Recently, placenta growth factor was shown specifically to stimulate MAP kinase and mitosis in porcine aortic endothelial cells expressing Flt-1 (41). Furthermore, stable expression of native and point-mutated Flt-1 and/or KDR in fibroblasts and endothelial cells lacking the corresponding endogenous receptors has identified Flt-1 activation and heterodimerization with KDR as a require-

ment for the induction of mitogenesis by VEGF.2 Heterodimerization of receptor tyrosine kinases has been shown to be crucial for the intracellular signaling of transforming growth factor-β (42), as well as for PDGF receptor-α and PDGF receptor-β, that is dependent on the ligand isof orm (43, 44). Thus, one might hypothesize that heterodimerization of KDR/Flt-1 enables Flt-1 to be involved in signaling mitogenesis in HMEC, as reported in our study. The cellular function and mechanism of action of Flt-1 are not well understood. Recently, Flt-1 was shown to mediate chemotactic activity in monocytes in response to VEGF and placenta growth factor, and to stimulate tissue factor expression in monocytes and endothelial cells (45). Recruitment of monocytes/macrophages is believed to play an important role in angiogenesis and wound healing (46). Tissue factor expression in endothelial cells has been shown to augment VEGF expression and to correlate with the switch to the malignant and angiogenic phenotype of breast carcinomas (47).

Targeted disruption of Flt-1 was shown to result in the formation of dysfunctional vascular tubes, an indication that Flt-1 could function in endothelial cell-cell and/or cell-matrix interactions (48). Flt-1 might therefore, at least in part, mediate the roles suggested for SPARC in the regulation of wound healing (31), angiogenesis (49), and metastasis (50), as well as the regulation of cell adhesion and proliferation in vitro (51, 52).

Differential regulation of VEGF receptor levels under conditions associated with overexpression of VEGF and hypoxia has been reported in malignant tumors, as well as in wound healing and rheumatoid arthritis (53, 54). The expression of Flt-1 has been shown to be selectively induced, and that of KDR diminished, in skin explant cultures and in human dermal endothelial cell monolayers under hypoxic conditions (55). Furthermore, VEGF present in the media conditioned by various cancer cells grown under hypoxic conditions was reported to augment the expression of Flt-1 mRNA and protein, but not KDR, in human umbilical vein and lung microvascular endothelial cells (56). However, by reverse transcription-polymerase chain reaction analysis of mRNA from HMEC grown under normoxic conditions, we were unable to show significant changes in either Flt-1 or KDR receptor levels in the presence of VEGF and SPARC, relative to those assayed from cells cultured with VEGF alone (data not shown).

We also observed that binding of VEGF to receptors on HMEC was reduced only 70% by SPARC or peptide 4.2 at concentrations sufficient to abolish VEGF-induced DNA synthesis (Fig. 3). Additional mechanisms distal to receptor activation are needed to account for the total inhibition of the proliferative effect of VEGF by SPARC. The 16-kDa N-terminal fragment of prolactin has been shown to block the mitogenic action of VEGF distal to autophosphorylation of KDR and proximal to activation of MAPK (29). In our studies, SPARC and peptide 4.2 resulted in a significant reduction of VEGF-stimulated Erk1 and Erk2 phosphorylation without a detectable change in the level of KDR phosphorylation (Fig. 5). It is plausible that VEGF-stimulated MAPK activation in HMEC is induced by a different pathway that is susceptible to SPARC. Cross-talk and synergism between growth factor-mediated signaling and integrin-mediated MAPK activation have been reported (57). In human umbilical vein endothelial cells, VEGF has also been implicated in the phosphorylation of focal adhesion kinase and paxillin (58), two proteins typically, but not exclusively, phosphorylated upon integrin activation. Our results could reflect involvement of signaling proteins upstream of MAPK that are not dependent directly on the activation of KDR. However, these speculations are at this point tentative.

2 T. Quinn, personal communication.
Expression of SPARC is increased during tissue remodeling, and SPARC is susceptible to degradation by a variety of matrix metalloproteinases (59, 60). Digestion of SPARC by plasmin produces several peptides, including one or more from domain IV, that have biological activity at the concentrations used in the present study (28, 49, 61). Since we have shown that peptide 4.2 mimics the effect of native SPARC and competes with SPARC for binding to VEGF, domain IV is an active site for the interaction of SPARC with VEGF. The coincident location of SPARC and VEGF in the developing mouse brain indicates a potential for interaction between the two proteins. In atherosclerotic tissue, the expression of VEGF (62) and SPARC (6) was also shown to be increased, and in some cells, coincident. SPARC might therefore modify the effects of VEGF under pathologic conditions.

Since mitogens act in concert with their cellular and extracellular environments, tight regulation of their potent action is necessary. It has been suggested that matricellular proteins function in part to control the availability as well as the activity of certain growth factors (4). In this study, we have shown that extracellular SPARC is an efficient modulator of VEGF activity at several different levels: 1) direct binding and thus reducing the available pool of ligand for interaction with its receptors, 2) efficient inhibition of the activation of Flt-1, and 3) inhibition of MAPK activation induced by VEGF.

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