

Neuroblastoma Angiogenesis Is Inhibited with a Folded Synthetic Molecule Corresponding to the Epidermal Growth Factor-Like Module of the Follistatin Domain of SPARC

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ABSTRACT

Secreted protein acidic and rich in cysteine (SPARC) is a multifunctional matricellular glycoprotein. *In vitro*, SPARC inhibits the proliferation and migration of endothelial cells stimulated by growth factors and induces endothelial cell apoptosis. We previously showed that SPARC also inhibits angiogenesis *in vivo* and impairs the growth of the pediatric tumor neuroblastoma (NB). SPARC comprises three domains that are independently folded by a complex pattern of disulfide bonds and have a high degree of structural conservation. In this study, separate modules of the SPARC domains were synthesized as cysteine-linked peptides and tested for their ability to inhibit angiogenesis. Peptide FS-E, representing the epidermal growth factor (EGF)-like module of the follistatin (FS) domain, did not cause endothelial cell apoptosis but strongly inhibited basic fibroblast growth factor (bFGF)-induced endothelial cell migration with an $ED_{50} = 10$ pmol/L. *In vivo*, peptide FS-E blocked bFGF-stimulated angiogenesis and neovascularization induced by NB cells. The EGF-like conformation was essential for peptide FS-E function because reduction of its two disulfide bonds completely abrogated peptide activity. Peptides FS-K and EC-N, corresponding to part of the Kazal module of the FS domain and the conserved α -helix in the extracellular calcium-binding domain, respectively, had minimal to no inhibitory activity. Our data show that the EGF-like module of the SPARC FS domain is angiosuppressive, and its structural conformation is critical for antiangiogenic activity.

INTRODUCTION

Secreted protein acidic and rich in cysteine (SPARC) also known as osteonectin and BM-40, is a matricellular glycoprotein involved in diverse biological processes (1, 2). Although the precise mechanisms by which SPARC modulates cell-matrix interactions remain unclear, its antiproliferative effect appears to be mediated, at least in part, by its ability to regulate the activity of certain growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF)₁₆₅, and basic fibroblast growth factor (bFGF; ref. 2). In laboratory studies, SPARC inhibits endothelial cell migration (3, 4), induces endothelial cell apoptosis (5), and potentially suppresses angiogenesis in the rat corneal assay (5). However, the role of endogenous SPARC in tumor angiogenesis and growth remains unclear. In some types of cancer, high levels of SPARC expression correlate with disease progression (6, 7). In contrast, antitumor effects have been seen in ovarian, pancreatic, and breast cancer and neuroblastoma (NB; refs. 5, 8–10).

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SPARC is a member of the follistatin (FS)-related protein family. The mature human protein consists of 286 amino acids and is divided into three distinct domains. The unstructured NH₂-terminal acidic (AC) domain (Ala1-Glu52) binds several calcium ions with low affinity and mediates the interaction with hydroxyapatite. The FS domain (Asn53-Pro137) folds into an elongated nonglobular structure assembled from two weakly interacting modules, each stabilized by a complex network of internal disulfide bonds. The NH₂-terminal module of the FS domain is a β -hairpin structure that is highly twisted by disulfide bonds linking cysteines 1 to 3 and cysteines 2 to 4. This region of SPARC has a high degree of structural homology to the epidermal growth factor (EGF)-like domain of the blood coagulation factor IX because of the highly conserved distribution of disulfide bonds (11). The FS domain COOH-terminal module consists of a pair of antiparallel α -helices connected to a small three-stranded antiparallel β -sheet with disulfide linkage between SPARC cysteines 5 to 9, 6 to 8, and 7 to 10. This region of SPARC is structurally similar to the Kazal family of serine proteases. The COOH-terminal extracellular calcium-binding (EC) domain (Cys138-Ile286) is globular and almost entirely α -helical. This domain contains two EF-hand motifs that bind Ca²⁺ with high affinity (11). A signaling peptide of 17 amino acids is removed during processing and is not a part of the mature SPARC protein.

To investigate whether these highly conserved structural modules are responsible for the antiangiogenic effects of SPARC, we synthesized peptides corresponding to the EGF and Kazal modules and the NH₂-terminal α -helix of the EC domain and tested their ability to inhibit angiogenesis. To maintain the structural integrity of the native modules, cysteines within the peptides were linked with disulfide bonds during the synthesis. Minimal to no inhibitory activity was observed with the Kazal module and the α -helix of the EC domain. In contrast, the EGF-like module peptide (FS-E) strongly inhibited endothelial cell migration *in vitro* and angiogenesis *in vivo*. Reduction of the two disulfide bonds in the FS-E peptide completely abrogated the angiogenesis inhibitory effects, indicating that structural conformation is critical for this biological activity.

MATERIALS AND METHODS

Peptide Synthesis. The amino acid sequence of human SPARC was compared with *Caenorhabditis elegans*, *Drosophila*, mouse, rat, and bovine SPARC, and peptides were designed to correspond to structurally conserved modules of the protein (Fig. 1). All of the peptides were synthesized at Alpha Diagnostics International (San Antonio, TX) using *fmoc/tboc* chemistry. The molecular mass of the peptides was checked by mass spectrometry, and high-performance liquid chromatography was performed to assess the purity of synthesis. Peptide FS-E (CQNHGKVCLEDENNTPMC) represents the EGF module of the FS domain (residues 55 to 76). It was folded into its native conformation by linking cysteines 1 with 3 and 2 with 4. Specific blocking groups protected each pair, and proprietary methods were used for disulfide bond formation. A nonfolded control peptide (nf-FS-E), in which the cysteines were not linked during the synthesis, and a scrambled peptide (sc-FS-E), which contains the same amino acids as peptide FS-E in a random order (TCHCM-CKQCGNHENPDNKEHVL), were made without special modifications. Pep-



Fig. 1. The amino acid sequence and domains of SPARC in distant species. The individual structural domains are shown above the alignment. Identical amino acid residues are shaded in yellow and include all of the cysteines of the mature protein. The EGF- and Kazal-like modules of the SPARC FS domain are aligned to domain I of human follistatin, EGF-like module of human blood coagulation factor IX, and domain III of turkey ovomucoid. The EF-hand calcium-binding module is compared with the EF consensus (11). SPARC peptides used in previous studies (12) are shown in the mouse SPARC sequence. Peptides used in the current study are indicated in the human SPARC sequence and named below their respective positions.

peptide FS-K (CPAIGFEFEKVA**SNDNKT*FDSSC), which corresponds to a highly conserved loop of the Kazal module of the FS domain (residues 84 to 106), was chemically folded by forming a disulfide bond between cysteines 6 and 8. Alanine was substituted for cysteine 7, which is not paired in the peptide, as indicated by the asterisk. Peptide EC-N (FPLRMRDWLKNVLTLYERDE) represents the highly conserved NH₂-terminal α -helix of the EC domain (residues 146 to 166). No special modifications were made during synthesis of the EC-N peptide. Endotoxin was measured in the peptide preparations as described previously (13).

Endothelial Cell Migration and Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assays. Endothelial cell migration assays were performed with human umbilical vein endothelial cells (National Cancer Institute Preclinical Repository, Bethesda, MD) and each of the synthetic SPARC peptides in EBM media (Cambrex Corporation, East Rutherford, NJ) containing 0.01% BSA as described previously (14). Serial dilutions of the peptides starting at 100 μ mol/L were assayed with or without 10 ng/mL bFGF (National Cancer Institute Preclinical Repository). At least three independent experiments were done for each peptide, and the data were normalized as a percent of maximal migration (100%) using the difference between bFGF-induced migration and background migration in the media without bFGF. Endothelial cell apoptosis was examined in bovine aorta endothelial cells (VEC Technologies Inc., Rensselaer, NY) treated overnight with serial dilutions of the peptides starting at 100 μ mol/L in serum-free DMEM containing 0.01% BSA as described previously (5).

Rat Corneal Assay. Hydron pellets (Interferon Sciences, New Brunswick, NJ) containing 100 μ mol/L peptide with or without 50 ng/mL bFGF were implanted into the corneas of anesthetized female Fischer 344 rats (Harlan, Madison, WI) as described previously (5). Pellets containing PBS with or without bFGF were used as controls.

Matrigel Assay. Matrigel assays were performed in 4- to 6-week-old homozygous athymic nude mice (Harlan). Mice were injected subcutaneously with 0.4 mL growth factor reduced Matrigel (Discovery Labware, Bedford, MA) containing 10 units/mL heparin, with or without 50 ng/mL bFGF. Peptides FS-E, sc-FS-E, nf-FS-E, or nf-FS-E peptide additionally treated with 1 mmol/L DTT at 100°C for 5 minutes were added to the Matrigel at 10 μ mol/L concentrations. Mice were sacrificed 7 days after the Matrigel injections. Gels were recovered by dissection, fixed in formaldehyde, and embedded in paraffin. A total of 4- μ m-thick sections were stained with anti-CD31 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:100 dilution. To evaluate vascular density, vessels were counted in five consecutive fields at $\times 200$ magnification, and the average microvessel density (MVD) per square millimeter was calculated. At least six plugs were analyzed for each peptide, and the statistical significance of the data was evaluated using the Student's *t* test. Additional experiments with dilutions of peptide FS-E ranging from 100 μ mol/L to 100 pmol/L were performed in at least five Matrigel plugs.

To visualize endothelial cells and pericytes, the Matrigel sections were double-stained with goat anti-CD31 (Santa Cruz Biotechnology) and mouse anti- α -SMA (DAKO Corporation, Carpinteria, CA) antibodies, followed by antigoat Texas Red and antimouse FITC-labeled secondary antibodies (Vector Laboratories, Burlingame, CA). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (Vector Laboratories). At least three areas of green and red fluorescence for each sample were photographed at $\times 100$ magnification and quantified with Image Pro software (Media Cybernetics, Silver Spring, MD), and the data were evaluated using the Student's *t* test.

Additional experiments were performed with Matrigel plugs containing NBL-W-N NB cells (15). Mice were injected with 0.5 mL of Matrigel containing 5×10^5 NB cells alone or combined with peptide FS-E or sc-FS-E at concentrations ranging from 0.1 μ mol/L to 10 μ mol/L. Matrigel plugs

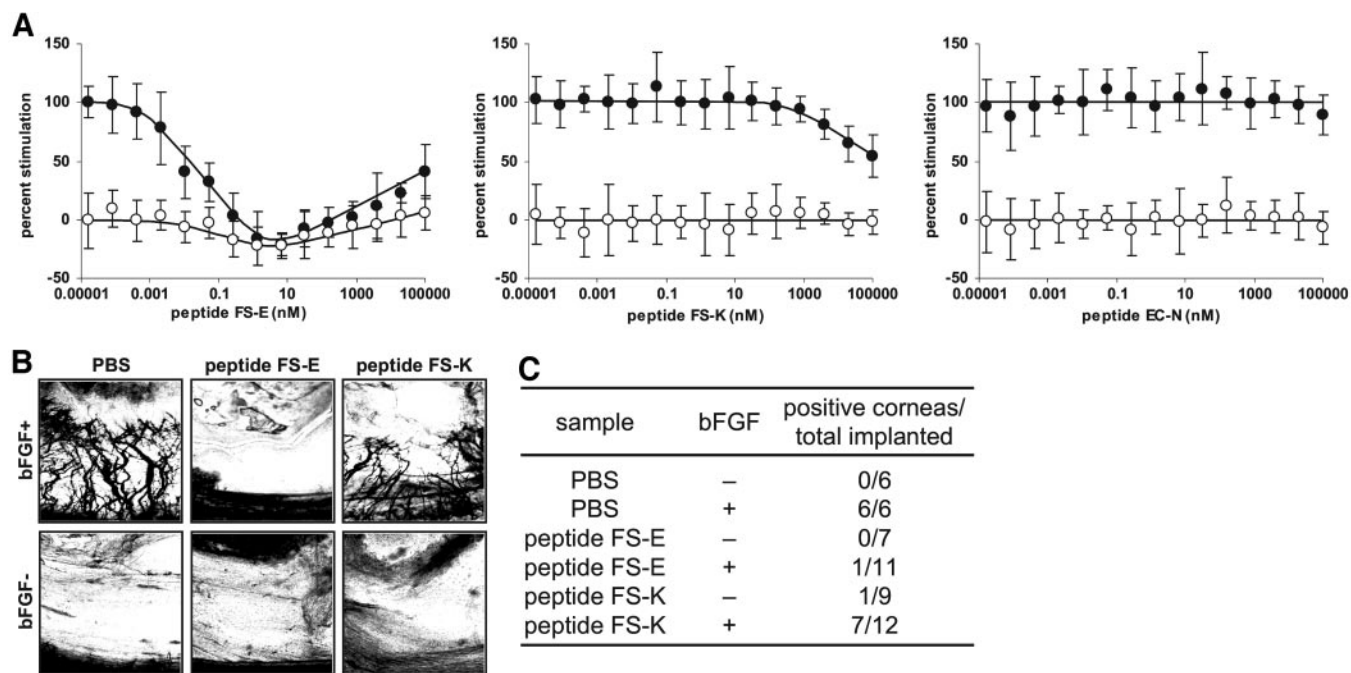


Fig. 2. Inhibitory activity of SPARC peptides. **A**, Peptide FS-E displayed dose-dependent inhibition of bFGF-stimulated endothelial cell migration with a half-maximal concentration of 10 pmol/L. Peptide FS-K was weakly inhibitory, whereas inhibition was not detected with peptide EC-N. Solid circles represent bFGF-stimulated migration. Open circles represent basal migration in the absence of activator. **B**, SPARC peptide activity in the rat corneal assay. Peptide FS-E blocked bFGF-induced angiogenesis, and weak inhibitory activity was observed with peptide FS-K (*top*). In the absence of bFGF, control media or the peptides did not induce angiogenesis (*bottom*). **C**, number of the positive corneas with dense capillaries reaching the pellet in the rat corneal assay with SPARC peptides in the presence and absence of bFGF.

without NBL-W-N cells, containing 50 ng/mL bFGF or PBS, served as controls. Animals were sacrificed 7 or 10 days after injection, and sections were processed to quantify vessel density. All of the animals were treated according to NIH guidelines for animal care and use, according to protocols approved by the Animal Care and Use Committee at Northwestern University.

RESULTS

FS-E, FS-K, and EC-N Peptides Correspond to Structurally Conserved Regions of SPARC. To investigate whether the structurally conserved modules within the FS and EC domains are responsible for the antiangiogenic effects of SPARC, peptides corresponding to the EGF-like module (FS-E), the Kazal module (FS-K), and the α -helix at the NH₂ terminus of the EC domain (EC-N) were synthesized (Fig. 1). The peptides were designed with relatively short sequences that could be folded into autonomous structural units by chemically linking internal cysteines to maintain the structural integrity of the modules. All of the peptide preparations were tested for endotoxin, and none was detected.

SPARC Peptide FS-E Inhibits Endothelial Cell Migration. Peptide FS-E strongly inhibited bFGF-stimulated endothelial cell migration with ED₅₀ of ~10 pmol/L (Fig. 2A), which is lower than 2 nmol/L ED₅₀ of the full-size SPARC (5). Significantly weaker inhibition of bFGF-stimulated endothelial cell migration was seen with peptide FS-K, and no inhibition was observed with peptide EC-N. Terminal deoxynucleotidyl transferase-mediated nick end labeling assays performed with peptides FS-E, FS-K, and EC-N showed that none of the SPARC peptides induced endothelial cell apoptosis (data not shown).

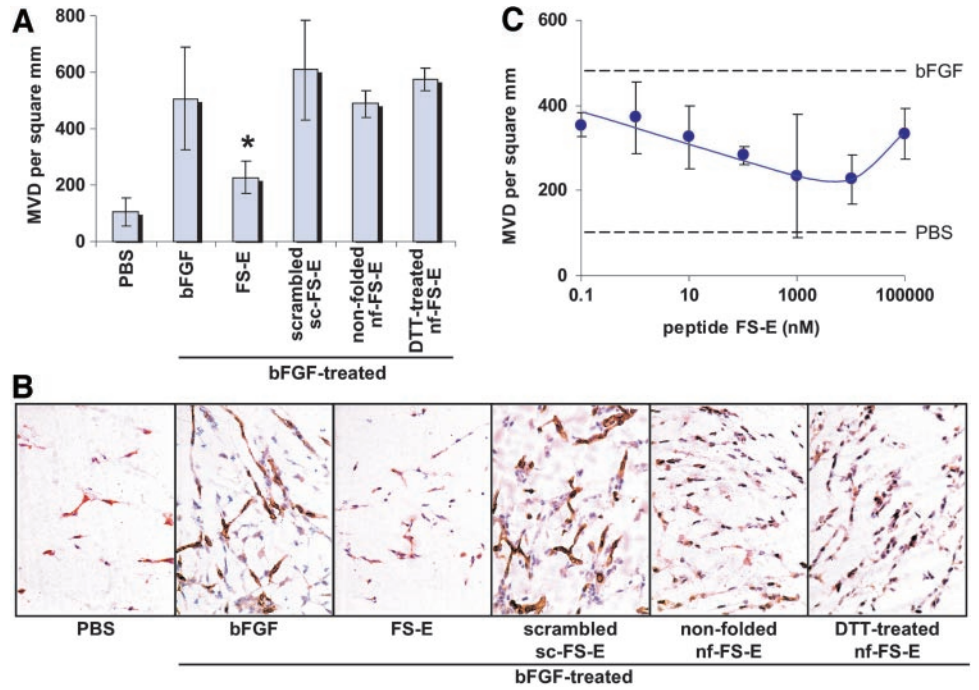
Peptide FS-E Inhibits Angiogenesis *In vivo*. Peptides FS-E and FS-K were tested for their ability to suppress angiogenesis *in vivo* in the rat corneal assay. Consistent with the endothelial cell migration assays, peptide FS-E potently inhibited bFGF-induced blood vessel growth *in vivo*, whereas only weak suppression was seen with peptide FS-K (Fig. 2B and C). Peptide FS-E also displayed significant angio-

suppressive activity in Matrigel plug assays. The MVD of Matrigel plugs with FS-E plus bFGF was 226 ± 57 compared with 507 ± 182 in experiments with bFGF alone ($P = 0.0011$; Fig. 3A and B). Structural conformation was essential for the antiangiogenic activity of the FS-E peptide because reduction of the two disulfide bonds completely abrogated its function. Compared with control Matrigel plugs containing bFGF alone, neovascularization was not inhibited in assays performed with the scrambled peptide sc-FS-E (MVD, 609 ± 177 ; $P = 0.27$), the nonfolded peptide nf-FS-E (MVD, 488 ± 46 ; $P = 0.38$), or the nonfolded peptide nf-FS-E with disulfide bonds additionally reduced by treatment with DTT (MVD, 576 ± 39 ; $P = 0.12$). The MVD of the control Matrigel plugs with PBS was 105 ± 48 (Fig. 3A and B).

To assess the relative potency of the FS-E peptide, we repeated the Matrigel plug assays using peptide concentrations ranging from 0.1 nmol/L to 100 μ mol/L. Neovascularization was inhibited at a broad range of concentrations, but the most potent effect was observed between 1 μ mol/L (MVD, 234 ± 164 ; $P = 0.0024$) and 10 μ mol/L (MVD, 226 ± 57 ; $P = 0.0011$). As shown in Fig. 3C, the concentration-dependent inhibition was biphasic in the Matrigel plug assays. A similar biphasic pattern of inhibitor activity was observed in endothelial cell migration assays performed with peptide FS-E (Fig. 2A) or full-length SPARC (5).

Matrigel Plugs Treated with Peptide FS-E Contain Decreased Number of Pericytes and Endothelial Cells. To further characterize the blood vessels in the Matrigel plugs, endothelial cells were labeled with red fluorescence using anti-CD31 antibody, and pericytes were visualized with green fluorescence using anti- α -SMA antibody. Blood vessel area was quantified with the Image Pro software (Media Cybernetics). Consistent with the calculated MVD, the blood vessel area was significantly decreased in the Matrigel plugs containing bFGF and FS-E peptide compared with the positive control with bFGF alone ($P = 0.0003$) and the plug containing bFGF with scrambled peptide sc-FS-E ($P = 0.001$; Fig. 4A and B). Relative size of a

Fig. 3. Inhibition of neovascularization by peptide FS-E in the Matrigel plug assay. **A**, average MVD of the sections of Matrigel plugs containing bFGF alone (positive control), or PBS (negative control), compared with Matrigel plugs with bFGF containing 10 $\mu\text{mol/L}$ peptide FS-E, scrambled peptide, nonfolded peptide, or peptide treated with DTT. Asterisks indicate statistically significant inhibition of neovascularization compared with the positive control Matrigel plugs containing bFGF alone ($P < 0.05$). **B**, representative CD31 staining of histologic sections from the Matrigel plugs; magnification, $\times 200$. **C**, concentration-dependent inhibition of neovascularization in Matrigel plugs by peptide FS-E.



single blood vessel was estimated using the ratio of the total area of the endothelial cell fluorescence to the MVD of the corresponding sample. In the Matrigel plugs containing bFGF and peptide FS-E, the relative size of the vessels (29.02 ± 9.25) was significantly smaller than the vessels in the Matrigel stimulated with bFGF alone (131.45 ± 58.70 ; $P = 0.001$) or bFGF and sc-FSE (87.63 ± 45.00 ; $P = 0.003$; Fig. 4C). Similar vessel size was detected in the FS-E Matrigel plugs and the PBS control (20.34 ± 22.08). We also calcu-

lated the ratio of pericytes to endothelial cells by measuring the areas of red *versus* green fluorescence. Similar pericyte to endothelial cell ratios were seen in the Matrigel plugs containing bFGF alone or bFGF combined with peptide FS-E or the scrambled peptide sc-FS-E (Fig. 4D). Although fewer vessels were detected in the Matrigel plug with PBS, the ratio of pericytes to endothelial cells was higher.

Peptide FS-E Inhibits Angiogenesis Induced by NB Cells. To study the impact of the peptide FS-E on angiogenesis induced by NB

Fig. 4. Quantification of endothelial cells and pericytes in Matrigel plugs. The endothelial cells in Matrigel plugs containing PBS, bFGF alone, bFGF with FS-E peptide, or bFGF with scrambled peptide sc-FS-E were labeled with red and pericytes with green fluorescence. **A**, photographs of the representative Matrigel plug sections taken at $\times 100$ magnification. **B**, relative quantity of endothelial cells and pericytes in the indicated Matrigel plugs estimated by calculating the area occupied by red and green fluorescence. **C**, relative blood vessel size. **D**, ratio of the pericytes to endothelial cells in indicated Matrigel plugs. Asterisks indicate statistically significant ($P < 0.05$) difference from bFGF control.

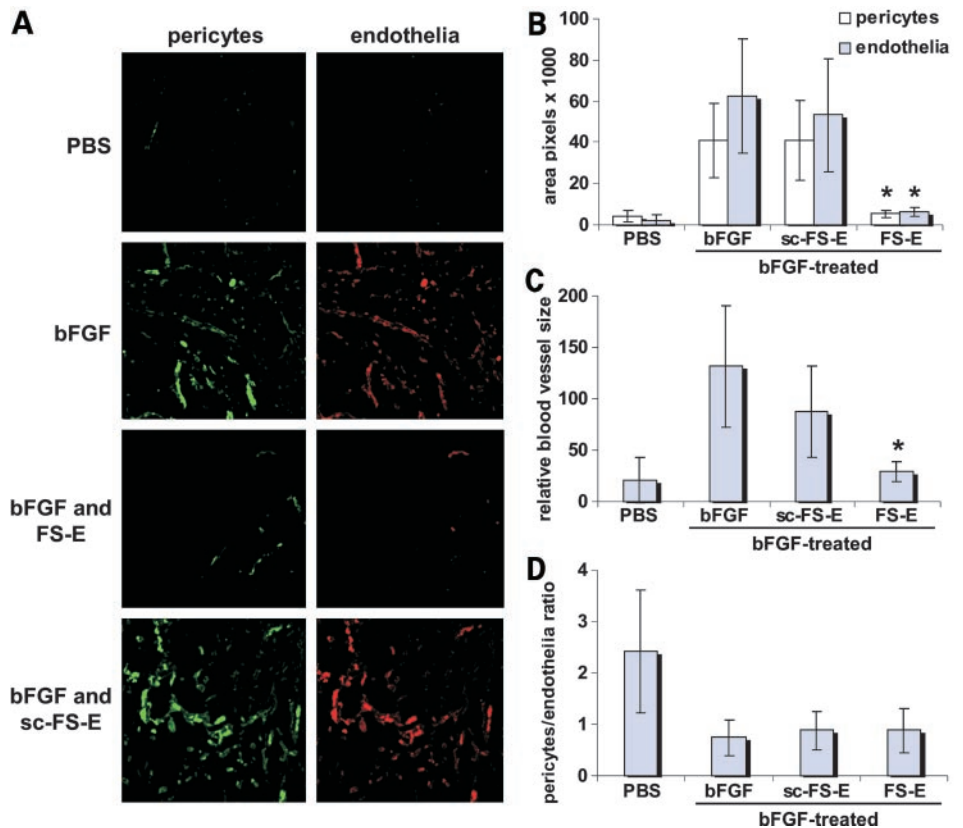
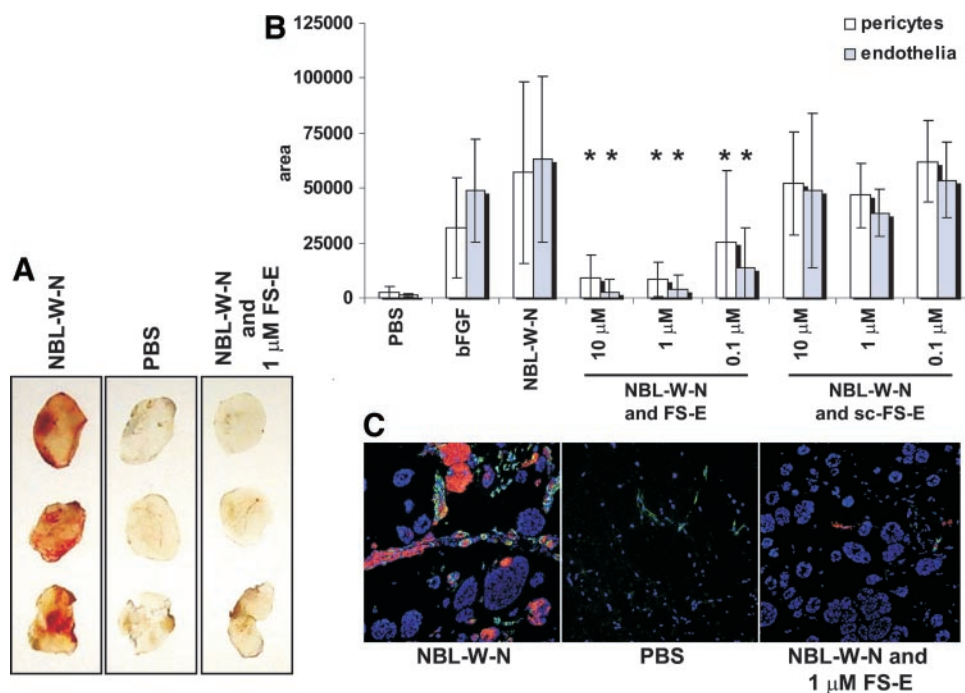


Fig. 5. Peptide FS-E inhibits neovascularization induced by NB cells. NBL-W-N cells were added to Matrigel plugs containing the indicated concentrations of the peptide FS-E, its scrambled analog sc-FS-E, or no peptide. Matrigel plugs containing only PBS or bFGF were used as negative and positive controls. A, gross appearance of Matrigel plugs containing NB cells alone, PBS, and NB cells combined with 1 μ mol/L FS-E peptide. B, area of fluorescence of endothelial cells and pericytes in Matrigel plugs containing NB cells with and without the indicated concentration of SPARC peptides. Asterisks indicate statistically significant inhibition ($P < 0.05$) versus NBL-W-N plugs. C, photographs of representative Matrigel plug sections taken at $\times 100$ magnification. Endothelial cells were labeled with red anti-CD31 antibody, pericytes with green anti- α -SMA antibody, and 4',6-diamidino-2-phenylindole staining was used to label nuclei.



cells, we added NBL-W-N NB cells to Matrigel plugs with peptide FS-E, sc-FS-E, or no peptide. In the absence of peptide, NBL-W-N cells potently induced neovascularization. Grossly the plugs with the NB cells without peptide appeared more hemorrhagic than the PBS controls and the plugs containing NB cells with FS-E peptide (Fig. 5A). Histologic analysis confirmed that the Matrigel plugs containing NBL-W-N NB cells or bFGF had significantly more blood vessels compared with the PBS controls ($P < 0.005$; Fig. 5B). NB-induced angiogenesis was inhibited by FS-E at concentrations ranging from 0.1 to 10 μ mol/L, although the lowest concentration was less efficient (Fig. 5B and C). In contrast, no inhibition of blood vessel formation was observed with the scrambled peptide sc-FS-E.

DISCUSSION

SPARC is a potent inhibitor of angiogenesis *in vivo*, and antitumor effects have been seen in many types of cancer including NB (2, 5). To identify the SPARC domains responsible for inhibiting NB tumor growth, we analyzed the antiangiogenic activity of three SPARC peptides designed to correspond to the highly conserved modules within the FS and EC domains. Our results show that peptide FS-E blocks endothelial cell migration in a biphasic manner, and it potently inhibits bFGF-stimulated angiogenesis and neovascularization induced by NB cells. Compared with control experiments, decreased numbers of endothelial cells and pericytes were seen in the Matrigel plugs containing peptide FS-E, and the size of the vessels was reduced. Reduction of the two disulfide bonds completely abolished the angiogenic inhibitory activity, indicating that structural conformation is required for FS-E function.

SPARC is proteolyzed in some cancer cells, and others have speculated that specific cleavage fragments may underlie the highly variable activities of SPARC in different types of tumors (16). In support of this hypothesis, synthetic peptides from distinct structural domains of SPARC have diverse biological activities (12). A fragment of the Kazal module (peptide 2.3; Fig. 1) stimulates endothelial cell proliferation and angiogenesis (17). This activity is contained within the short Cu^{2+} -binding motif KGHK, a morphogen/mitogen present in normal human plasma, which may be released from SPARC during

proteolytic matrix remodeling *in vivo* (18). In contrast, peptide 2.1, which spans a majority of the EGF-like module, and peptide 4.2, corresponding to the second EF-hand in the EC domain, inhibit endothelial cell spreading and proliferation (17). Peptide 2.1 differs from peptide FS-E by only three amino acids, although it lacks cysteine linkage. Thus, only a small fraction of the peptide may adopt a physiologically active conformation; therefore, relatively high concentrations of peptide may be required for biological activity.

Sage *et al.* (19) recently examined the angiogenic activity of three additional peptides representing motifs generated from cleavage of SPARC by matrix metalloproteinase-3. Peptide Z-1, which contains a Cu^{2+} -binding sequence KHGK and spans a similar region as peptides 2.1 and FS-E, exhibited a biphasic effect on endothelial cell proliferation *in vitro* and vessel growth in the quail chorioallantoic membrane assay, with higher concentrations showing inhibitory effects. Z-1 did not stimulate endothelial cell migration at any of the tested concentrations. In contrast, peptide Z-2, which corresponds to the EF hand-1 Ca^{2+} -binding motif, and peptide Z-3, which contains neither Cu^{2+} -binding nor EF hand-1 motifs, inhibited endothelial cell proliferation but stimulated migration. Thus, endothelial cell proliferation and migration were regulated in a mutually exclusive manner, emphasizing the broad range of effects different fragments of the SPARC protein can have on endothelial cells and vascular development.

More than 30 years ago, Dr. Judah Folkman hypothesized that tumor blood vessels might be effective therapeutic targets in cancer (20), and recent clinical trials have validated this theory (21). Our data indicate that low concentrations of a SPARC peptide designed to correspond to the EGF module in the FS domain potently inhibit endothelial cell migration *in vitro* and angiogenesis *in vivo* in a conformation-dependent manner. Peptides like FS-E may prove to be promising lead compounds in the development of antiangiogenic treatment strategies for neuroblastoma and other types of cancer.

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