Regulation of Gene Expression by SPARC during Angiogenesis in Vitro

CHANGES IN FIBRONECTIN, THROMBOSPONDIN-1, AND PLASMINOGEN ACTIVATOR INHIBITOR-1*

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growth of new vessels (angiogenesis) by their contribution to the synthesis and degradation of extracellular matrix (ECM)1 macromolecules (Folkman, 1984; Inger and Folkman, 1989; Liotta et al., 1991). The ECM, in turn, provides a variety of positive and negative signals to the resident endothelial cells. Confluent endothelial cells normally display low rates of replication and are noninvasive, properties that have generally been attributed to the presence of laminin and other components in the vessel wall (Furcht, 1986). Upon activation by phorbol esters or growth factors, endothelial cells secrete matrix-degrading enzymes and a variety of new extracellular gene products (Moscatelli et al., 1985; Inger and Folkman, 1988). Among ECM proteins, fibronectin (FN) and several types of collagen seem to play definitive roles (Risau and Lemmon, 1988; Inger and Folkman, 1988). Although changes in both the endothelial cell and its ECM are required for angiogenesis, many questions remain about how the extracellular environment functions to regulate specific features of cell behavior and the extent to which ECM signals are involved in the formation of new vessels.

The role of specific ECM proteins in angiogenesis has been particularly difficult to study in vivo due, in part, to our current lack of understanding of the molecular complexes that are formed in vivo and to problems in presenting these proteins in their native conformation. For example, the requirement of collagen synthesis for angiogenesis in vivo has relied on the use of inhibitors of protein processing which block collagen secretion but which can affect other cellular pathways as well (Maragoudakis et al., 1988; Inger and Folkman, 1988). Similarly, evidence that ECM molecules such as thrombospondin (TSP) can act as endogenous regulators of vessel growth has been derived from the identification of a truncated form of the molecule (Good et al., 1990). In view of these limitations, a number of systems have been developed in vitro that allow the dissection of singular events involved in vessel formation (Jaye et al., 1985; Montesano et al., 1988; Kubota et al., 1988). We have characterized specific clones of endothelial cells in which cords and patent tubes develop spontaneously under standard culture conditions, after the cells have synthesized their own ECM (Iruela-Arispe et al., 1991a, 1991b). This system differs from previous models of angiogenesis in vitro in that it does not rely on the use of exogenous growth supplements or matrix substrata. It also duplicates many of the activities of endothelial cell behavior observed

Vascular endothelial cells play a direct role in regulating

Angiogenesis in vitro, the formation of capillary-like structures by cultured endothelial cells, is associated with changes in the expression of several extracellular matrix proteins. The expression of SPARC, a secreted collagen-binding glycoprotein, has been shown to increase significantly during this process. We now show that addition of purified SPARC protein, or an N-terminal synthetic peptide (SPARC4-23), to strains of bovine aortic endothelial cells undergoing angiogenesis in vitro resulted in a dose-dependent decrease in the synthesis of fibronectin and thrombospondin-1 and an increase in the synthesis of type 1-plasminogen activator inhibitor. SPARC decreased fibronectin mRNA by 75% over 48 h, an effect that was inhibited by anti-SPARC immunoglobulins. Levels of thrombospondin-1 mRNA were diminished by 80%. Over a similar time course, both mRNA and protein levels of type 1-plasminogen activator inhibitor (PAI-1) were enhanced by SPARC and the SPARC4-23 peptide. The effects were dose-dependent with concentrations of SPARC between 1 and 30 μg/ml. In contrast, no changes were observed in the levels of either type I collagen mRNA or secreted gelatinases. Half-maximal induction of PAI-1 mRNA or inhibition of fibronectin and thrombospondin mRNAs occurred with 2–5 μg/ml SPARC and approximately 0.05 mm SPARC4-23. Strains of endothelial cells that did not form cords and tubes in vitro had reduced or undetectable responses to SPARC under identical conditions. These results demonstrate that SPARC modulates the synthesis of a subfamily of secreted proteins and identify an N-terminal acidic sequence as a region of the protein that provides an active site. SPARC might therefore function, in part, to achieve an optimal ratio among different components of the extracellular matrix. This activity would be consistent with known effects of SPARC on cellular morphology and proliferation that might contribute to the regulation of angiogenesis in vivo.

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during early angiogenesis, e.g. endothelial cell migration from a confluent monolayer, proliferation, organization of cords, and formation of lumina. Activities associated with late events in the stabilization of capillaries, such as the synthesis of laminin and the formation of a basement membrane, are not generally observed.

In previous studies we demonstrated that the morphogenesis of endothelial tubes was associated with changes in the expression of genes for specific extracellular proteins: an induction of type I collagen (Iruela-Arispe et al., 1991a), a decreased production of TSP, and an increased secretion of FN and SPARC (Iruela-Arispe et al., 1991a, 1991b). We have recently demonstrated that the production of type I collagen by these cultures is mediated by a de novo transcription of the α1(I) collagen gene in cells that formed unactivated cells in the same culture failed to transcribe detectable levels of this gene (Pouser et al., 1991). In a separate study, analysis of TSP protein demonstrated a specific interaction with cells forming capillary-like structures. The addition of anti-TSP antibodies to the cultures resulted in a specific increase in the number of cords. On the basis of these studies it was proposed that TSP might function to inhibit endothelial remodeling or to stabilize a noninvasive state (Iruela-Arispe et al., 1991c).

Thus, models of angiogenesis in vitro can provide systems whereby the roles of individual molecules can be studied.

In this study we address the role of SPARC (also known as osteonectin, BM-40, and 43K), a protein that is induced during the formation of endothelial cords in vitro. SPARC is a secreted Ca²⁺-binding glycoprotein associated with tissue growth and remodeling (Termine et al., 1981; Holland et al., 1987; Sage et al., 1989a; Kelm and Mann, 1990). In cultures of subconfluent proliferating endothelial cells, it has been shown to induce changes in cell shape (Sage et al., 1989b; Lane and Sage, 1990), delay entry into the S-phase of the cell cycle (Funk and Sage, 1991), and increase the production of endothelial type (type 1) plasminogen activator inhibitor (PAI-1) (Hasselaar et al., 1991). In addition, SPARC has recently been shown to modulate the activity of platelet-derived growth factor (PDGF) through a direct binding interaction (Raines et al., 1992).

We were interested in cultures of endothelial cells that formed cords and tubes (i.e. angiogenic cultures) to elucidate potential activities of SPARC in vivo. We show that addition of exogenous SPARC decreased the synthesis of FN and TSP but had no apparent effect on the expression of type I collagen. SPARC also stimulated the expression of PAI-1, a protein that can regulate the activation of latent TGF-β and the remodeling of matrix via its central role in the plasminogen activation pathway (Sato and Rifkin, 1989; Loskutoff et al., 1988). The response to SPARC required the activated morphology typical of angiogenic cultures of endothelial cells and appeared to be targeted specifically to cells involved in the formation of endothelial cords. Cultures of nonangiogenic BAE cells (i.e. cultures that did not form cords and tubes) showed markedly reduced responses. By using synthetic peptides corresponding to different regions of the molecule, we identified a peptide containing amino acids 4–23 of SPARC that had activities corresponding to those of the intact protein. A scrambled version of this sequence had no activity, and peptides from other regions of the protein were considerably less active, or inert, in these assays. We propose that the release of SPARC by endothelial cells could modulate the expression of ECM molecules in vivo in a paracrine and/or autocrine feedback manner. In view of the existing immunohistochemical evidence for the selective expression of FN, TSP, and plasminogen activators during angiogenesis in vivo,

this form of regulation might be important for progression of the invasive angiogenic phenotype.

EXPERIMENTAL PROCEDURES

Materials—All reagents were of analytical or molecular biology grade. Fetal calf serum (FCS) was obtained from HyClone (Logan, UT). Trypsin/EDTA and RNase-free phenol were from GIBCO-Bethesda Research Laboratories. Tissue culture plates were purchased from Costar (Cambridge, MA) and Thermobond™ coverslips were from Nunc (Naperville, IL). 4-15% gradient minigels were from Bio-Rad (Richmond, CA). 100 μg/ml of bovine serum albumin, 100 units/ml penicillin G, and 100 units/ml streptomycin sulfate. Cultures predisposed to cord formation (defined herein as angiogenic cultures) were selected and passaged with trypsin/EDTA (Iruela-Arispe et al., 1991a). Nonangiogenic BAE cells (i.e. cells that maintained a contact-inhibited monolayer and never formed cords or tubes), cultured under identical conditions, were used as controls. All clones were between passages 5 and 10.

Purification of SPARC, Peptide Synthesis, and Antibody Production—SPARC was purified from conditioned PYS-2 cell media as described previously (Sage et al., 1989a), and SPARC peptides were prepared as described (Lane and Sage, 1990). The sequences used in this study are derived from the published sequence of murine SPARC (Mason et al. 1986) and are named according to their location in the amino acid sequence; the N-terminal alanine of the mature secreted protein is designated as the 1st amino acid. Sequences of peptides discussed in the text are described in Fig. 1. Two scrambled peptides, containing amino acid residues between 4 and 23 of the secreted protein, were made by inversion of the native sequence and a redistribution of the glutamic acid residues. As noted in the text, we used both the scrambled 19mer (SPARC-19mer) and the 20-mer (SPARC-20mer) as control peptides in the experiments. Analysis of these sequences by the MacProMass™ program, developed by S. Vemuri and T. D. L. Beckman Research Institute, Duarte, CA), showed that the 19 mer predicted for each of the SPARC N-terminal peptide isomers (SPARC-19mer, SPARC-20mer, and SPARC-25mer) was 3.5.

Anti-SPARC antisera were produced against SPARC-19 (Lane and Sage, 1990). The antibodies were used in immunoblotting experiments.

FIG. 1. Sequences and location of SPARC peptides and their relation to functions demonstrated for the native SPARC protein. In A are listed the amino acid sequences and names of SPARC peptides used in the present study. Peptides were synthesized and purity monitored by reverse phase high performance liquid chromatography as described (Lane and Sage, 1990). In B is shown a schematic representation of the amino acid sequence of SPARC in conjunction with functional domains identified with synthetic peptides. In some experiments, SPARC-19mer, which lacked the glutamic acid residue (Q), was used in place of SPARC-19mer, to determine if activity was detected. a, numbers indicate amino acids; the N-terminal amino acid of the mature protein has been designated as 1. b, total number of amino acids. c, Lane and Sage, 1990. d, Funk and Sage, 1991.
Sage, 1990). An immunoglobin fraction from this serum specifically immunoprecipitated murine SPARC and has been shown to neutralize the anti-spreading activity of SPARC on endothelial cells. The antisera has low cross-reactivity with bovine SPARC. Rabbit anti-PAI-1 antiserum was a gift from Dr. David Loskutoff (Scripps Clinic and Research Foundation, La Jolla, CA). Rabbit anti-factor VII antigen (von Willebrand factor) was obtained from the Dako Corporation (Santa Barbara, CA). For immunohistochemical studies, the antisera were preincubated with FCS (1:10 dilution) for 24 h. This procedure effectively blocked the nonspecific reactivity of the anti-PAI-1 antiserum toward high molecular weight components in FCS and increased immunoreactivity toward PAI-1; this specificity was assessed by immunoblotting. The content of LPS in preparations used in this study was determined by a Limulus amebocyte lysate assay (Hasselaar et al., 1991).

**Biochemical Labeling of Secreted Proteins and Analysis by SDS-PAGE**—BAE cells were incubated in serum-free media for 12–24 h. Reagents were provided in fresh media every 24 h, and 50 μCi/ml of [2,3,4,5-3H]proline was added to each culture 12 h prior to collection of the media. For analysis of collagen, medium contained 50 μg/ml sodium ascorbate and 64 μg/ml β-aminopropionitrile furaminate. Conditioned media were clarified of cell debris by centrifugation, and protease inhibitors were added to a final concentration of 0.2 mM phenylmethylsulfonyl fluoride and 5 mM N-ethylmaleimide. For analysis of radiolabeled proteins, media were dialyzed against 0.1 N acetic acid and lyophilized.

**SDS-PAGE and Immunoblotting**—Proteins from conditioned media were lyophilized and resuspended in SDS-PAGE sample buffer (Laemmli, 1970). An aliquot was removed for scintillation counting, and samples were reduced in 50 mM dithiothreitol for 3 min at 95°C. Samples representing equivalent radioactivity were resolved on 4–15% SDS-polyacrylamide gels. Proteins were stained with Coomassie Brilliant Blue R-250 (0.25% in 45% methanol and 5% acetic acid). Destained gels were impregnated with ENHANCE® and dried before exposure to x-ray film at -70°C. For immunoblotting, conditioned media were diluted with SDS-PAGE sample buffer containing dithiothreitol and were heated at 95°C for 3 min. Equivalent volumes were applied to 4–15% gradient minigels. Proteins were transferred to nitrocellulose and blocked with MT buffer (PBS, pH 7.7, containing 1% nonfat dried milk and 0.05% Tween 20). Blots were incubated with respective antibodies for 2 h at room temperature. Antibody-antigen complexes were probed with 0.5 μCi/ml [3H]-protein A. Radiolabeled proteins were detected as autoradiographic images on x-ray film exposed at -70°C with two intensifying screens.

**58K cDNA and cDNA Probes**—Total RNA was isolated from cultures of BAE cells as described (Iruela-Arispe et al., 1991a). 5 or 10 μg of total RNA was denatured and resolved on 1.2% agarose gels containing 3% formaldehyde. To verify the integrity of the samples, gels were stained for 5 min with 0.5 μg/ml ethidium bromide in diethyrlactate-treated water, destained, and photographed. RNA was transferred to nylon membranes under vacuum and cross-linked to the support using ultraviolet irradiation (Stratalinker®). RNA was transferred to nylon membranes under vacuum and cross-linked to the support using ultraviolet irradiation (Stratalinker®). RNAs were prehybridized for 16 h at 42°C in PH buffer (50% deionized formamide, 30% 2X standard saline citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50 mM sodium phosphate, 4% 50 X Denhardt’s solution (1 X Denhardt’s is 0.2 μg/ml Ficoll, 0.2 μg/ml polyvinylpyrrolidone, and 0.2 μg/ml bovine serum albumin), and 10 μg/ml yeast total RNA). Hybridization occurred under the same conditions in the presence of 10°C/ml heat-denatured [32P]-cDNA probe. Final washing of hybridized RNA was performed in 0.1 X SSC, 0.1% SDS at 65°C. In the case of the PAI-1 probe, transfers were washed at 0.6 X SSC, 0.1% SDS at 65°C. The probes included: 1) a 1.3-kb fragment of human TSP-1 cDNA (Kobayashi et al., 1986), 2) a 557-bp fragment of mouse SPARC cDNA (Mason et al., 1986), 3) a 2.2-kb fragment of human FN cDNA (Segkiguchi et al., 1984), 4) a 1.1-kb fragment of human α1(I) collagen cDNA (Chu et al., 1982), 5) a 3-kb fragment of human PAI-1 cDNA (Norr et al., 1986; Sawdey et al., 1991a) or a 290-bp fragment of 0.2 μg/ml bovine ribosomal 28 S cDNA (Iruela-Arispe et al., 1991b). The 28 S probe was used to normalize the quantity of RNA applied to gels. Autoradiographic images were scanned with a Du-70 spectrophotometer (Beckman, Fullerton, CA) equipped with a film-scanning accessory.

**Immunohistochemistry**—BAE cells were grown on plastic coverslips until cords were clearly visible. Cultures were transferred to DMEM lacking FCS for 24 h and were subsequently treated with SPARC or SPARC peptides under serum-free conditions. After 20 h, cultures were washed 2 X with DMEM and fixed for 5 min with methanolic Carnoy’s solution (30% methanol, 60% chloroform, 10% acetic acid). Fixed cell preparations were blocked in PBS, pH 7.65, containing 1% normal goat serum and were incubated with adsorbed anti-PAI-1 antiserum (1:250 final concentration, by volume) or anti-factor VII antigen antiserum (1:100 final concentration). Specific labeling was detected with biotinylated goat-anti rabbit IgG secondary antibody, avidin-biotin-peroxidase, and diaminobenzidine, as specified by the manufacturer (Vector Laboratories, Inc., Burlingame, CA). Preparations were photographed under bright field optics with a Zeiss photomicroscope equipped with a x 25 planapochromatic objective.

**SDS Substrate Gels for Analysis of Proteases**—Conditioned media were subjected to substrate gel electrophoresis on 10% SDS-polyacrylamide gels containing 0.1% gelatin or casein (Herron et al., 1986; Unemori and Werb, 1986). Media were mixed with SDS-PAGE sample buffer lacking dithiothreitol and urea, and samples were resolved at 15 mA/gel. SDS was removed by washing the gels in 2% Triton X-100 (twice for 15 min). Gelatinase activity was initiated and developed by incubating the gels in Tris-Ca2+ buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl2, 0.02% NaNO3) for 36 h at 37°C. Gels were fixed and stained in 0.25% Coomassie Blue for 1 h and were subsequently destained. Gelatin-degrading enzymes were identified as clear zones on a blue background.

**RESULTS**

Three independent strains of BAE cells that exhibited angiogenesis in vitro (termed angiogenic cultures) were used in these experiments; each culture displayed morphological and biosynthetic phenotypes similar to those described in previous studies (Iruela-Arispe et al., 1991a, 1991b). After passage, angiogenic BAE cells formed confluent monolayers within 2 days, and cord-like structures were apparent by 7 days (Fig. 2A (C, control)). In the presence of 1% FCS, the addition of up to 40 μg/ml SPARC (Fig. 2A (SP, SPARC)) had no visible effect on the overall number of endothelial cords. Higher concentrations of the protein promoted an
obvious change in morphology that was typical of the rounded phenotype described elsewhere with respect to nonangiogenic BAE cells. In addition, SPARC peptides at concentrations of 0.1–0.4 mM, or anti-SPARC 4.2R IgG at approximately 150 μg/ml, caused no apparent morphological alterations in these cells (data not shown). Some experiments were carried out in the absence of serum. Under these conditions, individual peptide preparations would occasionally result in altered cellular morphologies; however, the results were inconsistent and did not correlate with the changes in gene expression described below.

To analyze the biosynthetic profile of the cells in the presence or absence of exogenous SPARC, [3H]proline-labeled culture media proteins were collected and analyzed by SDS-PAGE. As shown in Fig. 2B (lane C), cultures which did not receive SPARC exhibited a synthetic profile characteristic of angiogenic endothelial cells (Irulari-Arispe et al., 1991a). This secretory phenotype included high levels of FN (240 kDa), type I collagen (differential processing results in several bands between 100–200 kDa), and SPARC (40–43 kDa). After the addition of SPARC, there was a marked reduction in radio-labeled FN and an induction of a protein migrating at 46 kDa (Fig. 2B (lane designated SP)). The identity of the 240-kDa protein as FN was verified by immunoprecipitation and immunoblotting (not shown). The 46-kDa protein was identified as PAI-1, a protein previously shown to be induced by SPARC in subconfluent, but not in confluent cultures of nonangiogenic endothelial cells (Hasselaar et al., 1991). There was no apparent change in the production of SPARC, or of collagen, observed in these gels.

RNA was isolated from both angiogenic and nonangiogenic (control) cultures after treatment with native SPARC or with synthetic peptides derived from the amino acid sequence of murine SPARC. Initially, preparations of total RNA isolated from angiogenic cultures were probed with cDNAs corresponding to FN and PAI-1. SPARC and SPARC 4.2 caused a significant inhibition of FN mRNA by 24 h. Consistent with the results from protein gels, PAI-1 mRNA was induced and FN mRNA was diminished within 24 h. When these blots were probed with cDNAs corresponding to other ECM gene products, we saw no effect on the levels of α1(I) collagen mRNA. However, TSP mRNA decreased with kinetics similar to those of FN. In Fig. 3B, representative RNA transfers show the effects of SPARC, SPARC 4-23, and SPARC 24-273 on the expression of FN and PAI-1 mRNA in angiogenic BAE cell cultures. Addition of 20 μg/ml SPARC (Fig. 3B (lane 2)) caused a decrease in the level of FN mRNA at 24 h, which was more pronounced after 48 h (lane 6). PAI-1 mRNA was induced within 24 h (Fig. 3B (lane 2)) and was sustained over a period of 48 h (lane 6). The peptide SPARC 4-23 had activity similar to that of native SPARC (Fig. 3B (lanes 3 and 7)). Treatment with this peptide resulted in a decrease in mRNA for FN and, concomitantly, an increase in that for PAI-1. By comparison, peptide SPARC 24-273 from the C terminus of SPARC had no activity in these assays (Fig. 3B (lanes 4 and 8)).

Nonangiogenic BAE clones, those that did not form cords, had a markedly reduced response to exogenous SPARC or SPARC peptides. Under similar conditions, nonangiogenic cells became contact-inhibited and formed a typical cobblestone-like monolayer within days of plating. The confluent nonangiogenic BAE clones expressed low to undetectable levels of TSP, FN, and PAI-1 mRNA and responded to exogenous SPARC with a markedly attenuated induction of PAI-1 as described previously (Hasselaar et al., 1991). In Fig. 3A is shown RNA isolated from confluent nonangiogenic cultures. Control cultures expressed low levels of PAI-1 mRNA (Fig. 3A (lane 1)) and undetectable levels of FN (not shown). Treatment with 30 μg/ml SPARC elicited a marginal increase in PAI-1 mRNA after 24 h (Fig. 3A (lane 2)).

Addition of varying amounts of SPARC protein to angiogenic cultures showed that the response seen in Fig. 3B was dose-dependent (Fig. 4). Cultures were grown for 24 h in...
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serum-free media before treatment with SPARC. Levels of mRNA for TSP and FN decreased concurrently after addition of SPARC; significant diminution occurred in the presence of as little as 1 μg/ml (30 nM) SPARC. Quantitation of the RNA hybridization signals from these transfers revealed that TSP mRNA decreased approximately 5-fold, and that for FN, 4-fold, after the addition of 10 μg/ml SPARC. In contrast, levels of PAI-1 mRNA increased 4-fold, and that for FN, 1.9-fold after the addition of 10 μg/ml SPARC. Preincubation of the SPARC preparation with a 10-fold excess (by weight) of neutralizing anti-SPARC IgG resulted in a 50–60% recovery of FN and TSP mRNA levels and inhibited the induction of PAI-1 mRNA by a similar amount, compared with controls (not shown).

Experiments were conducted in the presence of peptides representing several domains of the SPARC protein; these results have been presented in Table I. Under serum-free conditions, SPARC and SPARC(4-23) inhibited the production of FN and TSP mRNA by 70 and 65%, respectively, within 24 h. After 48 h of continuous treatment, cultures maintained a reduced production of FN and TSP mRNA; however, we were concerned about the effects of extended culture in the absence of serum. To confirm that the results obtained under serum-free conditions were valid, we conducted parallel experiments in 1% FCS to approximate conditions that are more compatible with long-term viability of these cultures. Culture in 1% FCS produced results similar to those conducted in the absence of FCS. After 48 h of continuous treatment with SPARC in the presence of FCS, levels of TSP mRNA declined to 12% of control levels. It was interesting that levels of TSP mRNA in cultures treated with SPARC(4-23) appeared to recover by 48 h. Treatment of cultures with other peptides, including a scrambled isomer of SPARC(5-23) (SPARC(5-23scr)), had no significant effect on the production of any mRNA for which we probed (Table I, and data not shown). In SPARC(5-23scr), the charge and amino acid composition of the native peptide are conserved, but the sequence is altered (see Fig. 1A and “Experimental Procedures”).

Results presented in Fig. 5 (A and B) show a dose dependence in the responses of FN and PAI-1 mRNA after treatment of angiogenic BAE cells with peptide SPARC(4-23). PAI-1 mRNA increased 5-fold after 24 h with 0.1–0.2 mM peptide. The response of PAI-1 mRNA was saturable, and half-maximal induction occurred at approximately 0.05 mM. Half-maximal inhibition of FN mRNA was also seen at approximately 0.05 mM SPARC(4-23) and maximal inhibition occurred at approximately 0.2 mM. As shown with endothelial cells exposed to native SPARC (Fig. 4), FN and PAI-1 mRNA responded in a reciprocal fashion to SPARC(4-23). In addition, the half-maximal response of both FN and PAI-1 mRNA occurred at a similar dose of SPARC (2–5 μg/ml; 0.15 μM) and of SPARC peptide (0.05 mM). The scrambled isomer (SPARC(4-23scr)) had no apparent effect on the steady-state levels of FN or PAI-1 mRNA (Fig. 5C) or TSP mRNA (not shown).

Because of the relevance of plasminogen activators to various aspects of angiogenesis and tissue homeostasis, we continued to investigate the induction of secreted PAI-1 protein by SPARC in angiogenic cultures of BAE cells. An antisera which recognizes bovine PAI-1 was used to probe blots of protein from media conditioned by cultures of angiogenic and nonangiogenic BAE cells. In accord with our analysis of PAI-1 mRNA, confluent nonangiogenic BAE cells expressed very low to undetectable levels of PAI-1 protein which increased only slightly in response to treatment with SPARC (Fig. 3A and Hasselaar et al., 1991). In contrast, cultures of angiogenic BAE cells secreted low but detectable levels of PAI-1 protein which were increased considerably by treatment with SPARC. Fig. 6A is an immunoblot of PAI-1 secreted by angiogenic BAE cells cultured in the presence of increasing amounts of SPARC protein. Addition of exogenous SPARC correlated with the release of an immunoreactive protein that migrated with an apparent molecular weight of 46,000. Treatment of cultures with SPARC(4-23) also stimulated the secretion of PAI-1 protein, whereas peptides derived from other domains of

| Peptide name | Probe | mRNA levels* |
|--------------|-------|--------------|
| SPARC Fb     | 24 h (−FCS) 48 h (−FCS) 48 h (+FCS) |
| TSP          | 0.27 ± 0.04 0.62 ± 0.23 0.30 ± 0.00 |
| PAI-1        | 0.54 ± 0.02 0.39 ± 0.13 0.12 ± 0.01 |
| SPARC(4-23)  | 0.25 ± 0.23 0.24 ± 0.21 0.46 ± 0.06 |
| FN           | 0.39 ± 0.19 0.53 ± 0.01 0.24 ± 0.81 |
| TSP          | 0.27 ± 0.01 0.66 ± 0.08 0.64 ± 0.59 |
| PAI-1        | 1.00 ± 0.88 1.62 ± 0.10 3.28 ± 0.77 |
| SPARC(5-23scr) FN | 1.00 ± 0.88 0.70 ± 0.15 |
| TSP          | 1.94 ± 0.34 1.45 ± 0.60 3.27 ± 0.50 |
| PAI-1        | 1.00 ± 0.88 1.62 ± 0.10 3.28 ± 0.77 |
| SPARC(5-23scr) FN | 1.00 ± 0.88 1.62 ± 0.10 3.28 ± 0.77 |

* Numbers represent the average value ± S.D. from duplicate cultures. Values are normalized to a control (PBS) set at 1.00 and thus represent an arbitrary scale.

** ND, not determined.

![Fig. 5. Dose-dependent response of angiogenic BAE cells to SPARC(4-23).](image)
SPARC had significantly lower, or undetectable, activity (Fig. 6B). Different clones of angiogenic BAE cells displayed variability in the levels of PAI-1 released in response to SPARC (compare Fig. 6A with 6B), although all clones were stimulated; this effect was not investigated further.

To assess which cells in the culture were responding to exogenous SPARC, we localized PAI-1 protein by immunohistochemistry. Low levels of immunoreactive PAI-1 were associated with cords in cultures that were exposed to PBS (Fig. 7A (solid arrow)) or to a scrambled peptide (SPARC$_{1-28}$) (Fig. 7B); the protein was not detected in cells associated with the monolayer. Both SPARC and SPARC$_{4-23}$ induced a substantial increase in PAI-1 that was preferentially located in the endothelial cords (Figs. 7, C and D). By light microscopic examination the immunoreactivity appeared to coincide with the cytoplasm and the ECM of cells in cords. Cells associated with the monolayer of cultures treated with SPARC displayed a background level of reaction product and did not exhibit discernable levels of cytoplasmic immunoreactivity. The comparison of control (PBS or scrambled peptide) and SPARC-treated cultures provides an internal control for differences in path length, since the thickness of cords is unaltered in the presence of SPARC. Antisera against factor VIII antigen (Fig. 7, E and F) and against type III collagen (not shown) reacted with cells in the monolayer as well as with those in cords. The distribution or levels of these endothelial antigens did not appear to change following treatment with SPARC or SPARC peptides, and cells in cords were not stained selectively over those in the monolayer. By immunohistochemistry, these proteins do appear to concentrate slightly in cord-forming regions, a result of multiple cell layers.

Due to concern over contamination by endotoxin in certain reagents, and the known sensitivity of bovine endothelial cells to this compound, we investigated the effects of bacterial lipopolysaccharide (LPS) on the gene products analyzed in this study. LPS, the pharmacologically active endotoxin from the surface of Gram-negative bacteria, is a broad spectrum activator of BAE cells and a potent inducer of PAI-1 (Sawdey et al., 1989). First, we measured the levels of LPS in various preparations of SPARC and found a range between 0.005 and 0.02 ng of LPS/µg of protein. We then determined the levels of LPS required for activation of PAI-1 in BAE cells. As demonstrated by Hasselaar et al. (1991), removal of FCS from the incubations inhibited the response to LPS and did not affect the response to SPARC. In angiogenic cultures of BAE cells, induction of mRNA for PAI-1 required 0.5 ng/ml LPS in the presence of 1% FCS (not shown). Fig. 8 shows the dose-dependent activation of PAI-1 mRNA by LPS in the absence of FCS. The RNA was also hybridized with FN and TSP cDNA probes. Significant induction of PAI-1 required between 10 and 50 ng/ml LPS (10 ng of LPS represented 50–200 times the LPS content measured in 10 µg of SPARC protein). TSP levels were unaffected by LPS below 50 ng/ml, and higher amounts of LPS were slightly stimulatory (2-fold in the presence of 100 ng/ml LPS). At the same time, LPS had a minimally inhibitory effect on mRNA for FN (Fig. 8B). These results indicated that contamination of the SPARC preparations by LPS was unlikely to account for the observed changes in the synthesis of FN or TSP, since LPS had either little effect or an effect opposite to that seen with SPARC. In addition, contaminating levels of LPS were too low to account for the observed induction of PAI-1 by SPARC (Fig. 8 and
cultures, hybridized simultaneously with *"PcDNA probes for FN, TSP-1, and PAI-1. In were quantitated by scanning densitometry. Values are the average of the activities of several metalloproteases following SPARC or no detectable LPS activity.

Hasselaar et al., 1991). Peptides used in these assays contained no detectable LPS activity.

Experiments were also conducted to determine whether SPARC could induce the synthesis of gelatin-degrading metalloproteases by angiogenic cultures of BAE cells. We tested the activities of several metalloproteases following SPARC or LPS treatment of BAE cells. Fig. 9 shows an example of gelatin zymography conducted with conditioned medium from these cultures. Cultures in the presence of PBS synthesized a 64- and a 92-kDa gelatinase in the presence (Fig. 9 lane 1 and 4), SPARC, 20 μg/ml (lanes 2 and 5), or LPS, 50 ng/ml (lanes 3 and 6). The gelatinase activities were identified as metalloproteases by sensitivity to reduction, ethylenediaminetetraacetic acid, and 1,10-phenanthroline (not shown).

**DISCUSSION**

It is widely believed that the formation of new vessels by endothelial cells involves a series of events regulated by growth factors and ECM proteins. In a previous study, we proposed that SPARC might play an important role in this process, since it is synthesized at high rates when endothelial cells organize into cords and tubes in vitro (Iruela-Arispe et al., 1991a, 1991b). We were interested in extending this observation to delineate a mechanism by which SPARC could act during the formation of endothelial cords and to identify domains within the SPARC molecule that could be involved in this function. Because the acquisition of an angiogenic phenotype is associated with increased secretion of SPARC, we were interested in studying possible feedback pathways that could be potentiated by the enhanced availability of this protein during angiogenesis in vitro.

SPARC is a Ca**²⁺**-binding protein secreted by cultured endothelial cells isolated from normal blood vessels (Sage et al., 1981, 1984) and by a variety of cells in the vessel wall following vascular injury (Raines et al., 1992). When added to cultured cells, SPARC inhibited the spreading of endothelial cells on plastic and collagenous substrates (Sage et al., 1989b). Subsequently, active sites in the molecule were identified by the use of synthetic peptides. Anti-spreading activity was located at the N terminus (SPARC**₄₋₄₀**) and was blocked by N-terminal-specific antibodies (Lane and Sage, 1990). A portion of the C terminus, SPARC**₆₋₄₇₇**, also displayed anti-spreading activity and, in addition, competed for the binding of SPARC to collagens. These studies indicated that SPARC might function by blocking the interaction of cells with ECM, but the possibility of a direct action on cells mediated by surface receptors could not be ruled out. A recent study has demonstrated that SPARC increased the production of PAI-1 by subconfluent proliferating endothelial cells but had little effect on contact-inhibited nonangiogenic endothelial monolayers (Hasselaar et al., 1991). Since the induction of PAI-1 was apparently independent of overt changes in cell shape or adhesion, other mechanisms of signal transduction were implied.

In the present study we provide evidence that the synthesis of a small subset of ECM proteins, including FN, TSP, and PAI-1, can be modulated by SPARC and that the mechanism
of regulation includes alterations in the steady-state levels of the respective mRNAs, as well as in amounts of secreted protein. The effects were most pronounced in BAE cells in an activated state of cellular migration and remodeling, as typically seen in angiogenic cultures. The response was muted or absent in nonangiogenic (contact-inhibited) endothelial cell monolayers. In addition, neutralizing anti-SPARC peptide antibodies directed against amino acids 1-23 significantly diminished the effects of exogenous SPARC. However, our experiments with anti-SPARC antibodies have generally proven more difficult to control than those in which SPARC peptides were utilized. It is possible that the high levels of endogenous SPARC made by these cultures, or complexities in the experimental model not currently understood, contributed to these problems.

Both SPARC and the N-terminal peptide SPARC4-23, had similar activities with respect to the regulation of FN, TSP, and PAI-1, an observation supportive of previous data which indicated that the N terminus of the protein might contain a bioactive domain. In the present experiments, SPARC4-23 was approximately 300-fold less active as compared with the native protein. Loss of activity has been a consistent finding in a variety of studies on peptide homologs of active sites in large proteins (Lane and Sage, 1990 and references therein). Although it is not clear what variables are most important for the retention of optimal peptide activity, it is likely that peptide fragments lose conformational constraints contributed by neighboring and distant structures within multidomain proteins. To control for the effects of peptide sequence and concentration, we show that peptides from other regions of SPARC, and a scrambled version of the N-terminal sequence, had little or no activity.

To determine the location of cells responding to SPARC in angiogenic cultures, we investigated the production of PAI-1 protein by immunocytochemical methods. These experiments revealed that cells associated with cords selectively expressed PAI-1, a result consistent with the active remodeling that is characteristic of these cells. PAI-1 immunoreactivity associated with endothelial cords was enhanced after treatment of the cultures with exogenous SPARC. We found no evidence to suggest that cells in the monolayer contributed significantly to the production of PAI-1, in the presence or absence of exogenous SPARC. This observation is significant, because it is the cells that form cords and tubes, rather than quiescent cells in the monolayer, that produce SPARC in these cultures (Iruela-Arispe et al., 1991a). Endothelial cells involved in active remodeling might therefore produce proteins that selectively alter their extracellular environment and might in turn respond to this new environment in an autocrine manner. Other endothelial cells not directly involved in remodeling do not appear to respond. Although it is not clear why the cells in the monolayer were unaffected, it may be important that the responding cells were actively migrating and were not spread, a phenotype that is associated with a wide range of endothelial responses (Ingber et al., 1987; Ingber and Folkman, 1988; Canfield et al., 1990). Such differences might in fact be highly significant in regulating the extent of angiogenic phenomena in vivo.

FN and TSP are major secretory products of endothelial cells in culture (Sage et al., 1981; Raugi et al., 1982) and are important mediators of endothelial cell attachment (Murphy-Ullrich and Höök, 1988; Taraboletti et al., 1990). FN is present early in tissues undergoing angiogenesis and is thought to provide a provisional matrix for the migration of endothelial cells (Risau and Lemmon, 1986 and references therein). Synthesis of FN is increased in the presence of angiogenic factors (Jaye et al., 1985; Madri et al., 1988) and decreased by angiotropic steroids (Folkman and Ingber, 1987). Cell spreading on FN-coated plates resulted in elevation of cytoplasmic pH and increased rates of cell proliferation (Ingber et al., 1990). In addition, there is evidence that binding of FN to cells can activate cell surface Na+/H+ antiporters, a phenomenon that might explain some cellular responses to this protein (Ingber, 1990; Ingber et al., 1990). In the present study we demonstrated that exogenous SPARC inhibited the synthesis of FN mRNA and protein. Although it is not clear whether the activity of SPARC was direct or indirect, the functional significance of a decrease in extracellular FN could include the modulation of endothelial attachment, migration, and/or proliferation.

In contrast to FN, TSP does not appear to be associated with the earliest stages of endothelial budding but with more advanced capillaries, after basement membrane components have been deposited (O'Shea, 1987; O'Shea and Dixit, 1988). TSP has also been shown to inhibit endothelial proliferation (Bagavandoss and Wilks, 1990) and to block the mitogenic response of capillary endothelial cells to serum (Taraboletti et al., 1990). In culture, the biosynthesis of TSP is highest in endothelial cells that are prevented from spreading (Canfield et al., 1990) as well as in rapidly proliferating endothelial cells, but it is diminished as the cells reach confluence (Raugi et al., 1982; Mumby et al., 1984). Although the regulation of TSP has been studied in smooth muscle cells (Majack et al., 1985) and fibroblasts (Donoviel et al., 1990), information concerning its control in endothelial cells is minimal. Recently, we demonstrated that TSP has anti-angiogenic effects in our BAE cultures and might contribute to the stabilization of endothelial tubes (Iruela-Arispe et al., 1991c). In addition, a truncated form of TSP has been shown to suppress angiogenesis induced by basic fibroblast growth factor (Good et al., 1990). The present study shows that SPARC can functionally diminish mRNA for TSP in angiogenic BAE cells. As previously noted, SPARC and TSP inhibit the proliferation of endothelial cells in vitro (Funk and Sage, 1991; Taraboletti et al., 1990; Bagavandoss and Wilks, 1990); SPARC and TSP might therefore act cooperatively to regulate proliferative and adhesive properties of endothelial cells (reviewed in Sage and Bornstein, 1991). Additional members of the TSP family have recently been identified (e.g. Thbs2; Bornstein et al., 1991), but the expression of these gene products was not addressed in the present study.

The induction of PAI-1 by SPARC has several implications for hemostasis and tissue remodeling. PAI-1 is a specific and potent inhibitor of both urokinase and tissue-type plasminogen activators (Loskutoff et al., 1988) and therefore plays an important role in maintaining the nonthrombogenic properties of the vessel wall. Plasmin has been implicated as an activator of latent TGF-β (Sato and Rifkin, 1989; Lyons et al., 1990), and PAI-1 can inhibit the production of active TGF-β in cell culture (Sato and Rifkin, 1989). Because TGF-β is known to increase endothelial PAI-1 and decrease production of plasminogen activators (Roberts et al., 1988), an effective feedback loop might exist among these factors. Activated plasmin has a demonstrated proteolytic activity against SPARC (Sage et al., 1984) and several ECM components. By inhibiting plasminogen activators, PAI-1 could function to protect ECM proteins from these proteases (Sakse and Rifkin, 1988) and to stabilize cellular attachments to matrix (Ciambrone and McKown-Longo, 1990). In vivo, increases in protease inhibitors induced by SPARC could function to protect specific matrix components. PAI-1 itself, by inhibiting local matrix turnover, might also cause a graded
reduction in the angiogenic response.

ECM components act at many different levels to regulate angiogenesis. The binding of ECM to cell surface receptors can lead to the activation of a number of intracellular signal transduction pathways (Ingber and Folkman, 1989) and result in both direct and indirect modulation of cell shape, migration, and proliferative response. In addition, the stabilization or sequestering of specific growth factors by matrices of various compositions can strongly affect the distribution and activity of these factors (Rifkin and Moscatelli, 1989). Several growth factors that can potentially regulate angiogenesis include basic fibroblast growth factor (Folkman and Klagsbrun, 1987), TGF-β (Roberts et al., 1986), and platelet-derived growth factor-BB (PDGF-BB) (Fiegel et al., 1991; Madri et al., 1991). Although these morphoregulatory factors are not structurally related, they appear able to promote endothelial cell migration. They are also capable of promoting synthesis or turnover of ECM and are differentially sequestered by specific components of the ECM.

SPARC has demonstrated activities that are consistent with its classification as a morphoregulatory protein (Sage et al., 1989). Since it inhibits the spreading of endothelial cells and fibroblasts on collagen or plastic substrates (Sage et al., 1989b; Lane and Sage, 1990) and reduces the number of focal contacts made by BAE cells in culture (Murphy-Ullrich et al., 1991), SPARC could affect migratory or adhesive activities of the endothelium in vivo. It is not clear whether SPARC is a normal constituent of the ECM; however, the protein does interact with a variety of ECM components, a property that could regulate its distribution or stability. SPARC binds to several collagens, including types I, III, IV, and V (Termine et al., 1981; Sage et al., 1989b, Nieth et al., 1991) and, to a lesser extent, TSP and FN (Clezardin et al., 1988; Sage et al., 1989b). Recently, SPARC was also shown to bind the B chain of PDGF (Raines et al., 1992). Specific binding of PDGF-B isoforms would suggest a novel function for SPARC in the regulation of this potent angiogenic and growth-regulatory factor. Whereas aortic endothelial cells, such as those used in the present study, are not responsive to PDGF, capillary endothelial cells are stimulated to proliferate and migrate by PDGF-BB homodimers (Smits et al., 1989; Bar et al., 1989). It is therefore likely that endothelial cells derived from capillary beds would provide an optimal system to study the activities of both SPARC and PDGF-BB.

We suggest that endothelial cells, activated by specific stimuli, have the ability to regulate angiogenesis through their responses to both positive and negative feedback pathways. During angiogenesis, the invasive endothelial phenotype is initiated by the production of matrix-degrading proteases and diminution of specific protease inhibitors such as PAI-1 (Montesano et al., 1990). However, the production of FN, type I collagen, and other substrate macromolecules that are permissive for endothelial migration and proliferation is also required. The angiogenic cycle is completed when basement membrane components are assembled that stabilize capillary structure and inhibit further migration and proliferation. Although many of the functions proposed for SPARC remain unproven in vivo, activities attributed to the protein based on in vitro experiments are consistent with actions at sites of tissue remodeling. During angiogenesis, SPARC could function to regulate endothelial attachment and proliferation, possibly through the binding of specific growth factors. After the initiation of angiogenesis, SPARC could regulate the production or stability of ECM by modulating the synthesis of FN, TSP, and PAI-1.
Regulation of Extracellular Matrix Proteins by SPARC

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