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SPARC Antagonizes the Effect of Basic Fibroblast Growth Factor on the Migration of Bovine Aortic Endothelial Cells

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Abstract Migration of endothelial cells is requisite to wound repair and angiogenesis. Since the glycoprotein SPARC (secreted protein, acidic and rich in cysteine) is associated with remodeling, cellular migration, and angiogenesis in vitro, we questioned whether SPARC might influence the motility of endothelial cells. In this study we show that, in the absence of serum, exogenous SPARC inhibits the migration of bovine aortic endothelial cells induced by bFGF. Similar results were obtained from two different assays, in which cell migration was measured in a Boyden chamber and in monolayer culture after an experimental wound. Without bFGF, the migration of endothelial cells was unaffected by SPARC. The inhibitory effect of SPARC on cell motility was dose-dependent, required the presence of Ca^{2+}, was mimicked by synthetic peptides from the N- and C-terminal Ca^{2+}-binding domains of the protein, and was not seen in the presence of serum. Modulation of the activities of secreted and cell-associated proteases, including plasminogen activators and metalloproteinases, appeared not to be responsible for the effects that we observed on the motility of endothelial cells. Moreover, a molecular interaction between SPARC and bFGF was not detected, and SPARC did not interfere with the binding of bFGF to high-affinity receptors on endothelial cells. Finally, in culture medium that contained serum, SPARC inhibited the incorporation of [^{3}H]-thymidine into newly synthesized DNA, both in the absence and presence of bFGF. However, DNA synthesis was not affected by SPARC when the cells were plated on gelatin or fibronectin in serum-free medium. We propose that the combined action of a serum factor and SPARC regulates both endothelial cell proliferation and migration and coordinates these events during morphogenetic processes such as wound repair and angiogenesis.

Key words: angiogenesis, vascular biology, chemokinesis, extracellular matrix

The formation of new capillaries (angiogenesis), a process that occurs during tissue repair, wound healing, growth of malignant tumors, and embryonic development, requires both the migration and proliferation of endothelial cells. From attempts to identify factors that regulate angiogenesis in vitro and in vivo, basic fibroblast growth factor (bFGF) has emerged as a potent inducer of this process [Folkman, 1984; Montesano et al., 1986; Gospodarowicz et al., 1987]. In addition to soluble growth factors, the underlying extracellular matrix may have a regulatory effect on endothelial cell behavior [Kubota et al., 1988; Ingber and Folkman, 1989a,b]. We have previously identified SPARC (secreted protein, acidic and rich in cysteine) as one of the proteins that is upregulated during the spontaneous formation of endothelial tubes [Iruela-Arispe et al., 1991]. SPARC is a secreted, Ca^{2+}-binding glycoprotein that is associated with proliferation, morphogenesis, remodeling, and cellular migration [Mason et al., 1986a,b; Holland et al., 1987; Sage et al., 1989a,b]. Molecular cloning and protein sequence analysis have demonstrated that SPARC is identical to osteonectin, a major noncollagenous protein of bone [Termine et al., 1981; Romberg et al., 1985; Mason et al., 1986b; Findlay et al., 1988], to BM-40, a product of a basement membrane-secreting tumor cell line [Dziadek et al., 1986; Mann et al., 1987], and to an M_r 43,000 "culture shock" protein that was increased after endotoxin-mediated injury of endothelial cells [Sage et al., 1986]. From studies with bovine endothelial cells, fibroblasts, and

Abbreviations used: BAE, bovine aortic endothelial; bFGF, basic fibroblast growth factor; PAI-1, type 1 plasminogen activator inhibitor; PDGF, platelet-derived growth factor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

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smooth muscle cells, it was shown that SPARC elicited a rounded cellular morphology, inhibited the spreading of newly plated cells, and bound to several components of the extracellular matrix that included types III and V collagens [Sage et al., 1989b]. In recent studies with bovine aortic endothelial (BAE) cells, SPARC retarded cell-cycle progression by inhibiting DNA synthesis [Funk and Sage, 1991] and induced the expression of type 1 plasminogen activator inhibitor (PAI-1) [Hasselaar et al., 1991]. As an important regulator of plasminogen activator (PA) activity, PAI-1 controls locally many morphogenetic processes that require proteolysis. Therefore, it is possible that SPARC, as an antispreading factor, growth-regulatory protein, and/or inducer of PAI-1, could contribute to an environment that regulates cellular migration and remodeling.

In this report, we examined whether SPARC influenced the motility of BAE cells. Using both an in vitro wound model and a Boyden chamber assay, we found that the basal level of migration of BAE cells was unaffected by different concentrations of SPARC. However, SPARC inhibited the cellular migration that was induced by bFGF in a dose-dependent manner. The inhibitory effect of SPARC on migration required Ca^{2+}, could be attributed to the Ca^{2+}-binding domains of SPARC that were previously described as inhibitors of cell spreading [Lane and Sage, 1990], and was not observed in the presence of serum. Although levels of secreted PAs were diminished in the presence of SPARC, the expression of metalloproteinases and cell-associated PA was not altered. SPARC also inhibited BAE cell DNA synthesis in the presence of serum, as previously reported [Funk and Sage, 1991], but this activity was abrogated when serum was removed from the cultures. In summary, we propose that SPARC, in addition to controlling endothelial cell proliferation, is involved in the regulation of endothelial cell migration in remodeling tissues. Furthermore, we speculate that SPARC might regulate, in concert with bFGF and a factor that is present in serum, a reversible transition between cellular growth and migration.

METHODS

Purification of SPARC and Synthesis of SPARC Peptides

SPARC was purified from murine parietal yolk sac cells, bovine fetal calf ligament fibroblasts, and BAE cells as previously described [Sage et al., 1989b]. Bovine and mouse SPARC exhibited identical chromatographic properties and produced the same results when added to BAE cells. SPARC peptides from various domains of the SPARC molecule were synthesized and purified as described by Lane and Sage [1990]. Levels of endotoxin in the preparations were measured with the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA).

Cell Culture

BAE cells were cultured as previously described [Sage et al., 1989b]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (Flow Laboratories Inc., McLean, VA), 250 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin-G (Sigma), and 100 units/ml streptomycin sulfate (Sigma). Experiments were performed with 3 different strains of BAE cells between passages 4 and 10.

Migration Assays

In vitro wound assay. Confluent monolayers of BAE cells in 6-well tissue culture plates (Costar) were incubated in serum-free DMEM 4 days after the culture had reached confluence. After 24 h, the monolayer was partially denuded by a one-directional scrape with a rubber policeman. A mark was made with a sterile needle in the tissue culture plastic to facilitate localization of the same microscopic field during the course of the experiment. The cells were washed 3 times with serum-free DMEM, and 1 ml of serum-free DMEM was subsequently added to each well. Additions of phosphate-buffered saline (PBS), purified SPARC, and bFGF (Collaborative Research Inc., Bedford, MA) were performed as indicated. The cells were incubated at 37°C for 20 h in a humidified atmosphere containing 5% CO_2. The cultures were photographed at the beginning of the experiment and at several times during the incubation. Migration was quantitated by counting the number of cells that had migrated into the premarked denuded area, and by measuring the distance of migration in the same areas. These values were obtained by superposition of the photographic slides that were taken during the experiment. Accurate overlay was made possible by the needle markings. All experiments were performed 3 times in triplicate wells. Within each experi-

ment the values of control migration (no additions) were set at 100% (representing between 50 and 80 cells/field/20 h and between 65 and 90 μm/20 h). The motility values of cells that had been supplemented with bFGF and/or SPARC were then expressed as a percentage of control.

Boyden chamber assay. BAE cell migration was measured in a 48-well micro-Boyden chamber (Neuroprobe Inc., Cabin John, MD). Confluent monolayers of BAE cells were plated at confluent density (7×10^4 cells/cm^2) to minimize and standardize cell-substrate interactions. After 24 h the cells were washed twice with a 0.02% solution of EDTA and were detached by brief exposure (2–3 min) to 0.05% trypsin/0.02% EDTA. Trypsin was subsequently neutralized by adding DMEM/10% FCS to the BAE cell suspension. After centrifugation (1,200 *g* for 5 min), the cells were allowed to recover in DMEM/10% FCS for an additional hour. The cells were centrifuged again and resuspended in DMEM containing 0.25% ovalbumin (Sigma). Preparations of bFGF, SPARC, and SPARC peptides were diluted in DMEM/ovalbumin and were added to the lower wells of the Boyden chamber. Except for the experiments from Table I, all Boyden chamber experiments were performed in the absence of FCS. A fibronectin-coated polycarbonate filter containing 8 μm pores (Nucleopore Corp., Pleasanton, CA) was placed between the upper and lower wells. The filters were coated by immersion overnight in a solution of PBS containing 1 μg fibronectin/ml and were subsequently air-dried. Fifty microliters of the BAE cell suspension (2.5×10^5 cells/ml of DMEM containing 0.25% ovalbumin) was added to each well of the upper compartment. To assess the chemotactic and chemokinetic components of cell migration, bFGF was also added to the upper wells at a concentration equal to that present in the lower wells. The chamber was incubated at 37°C for 5 h in a humidified atmosphere containing 5% CO_2. At the end of the incubation period, the filters were removed, fixed, and stained with Diff-Quik (Baxter Healthcare Corp., McGaw Park, IL). The filters were mounted on glass slides (the side with the migrated cells faced down). The upper surface of the filters was wiped clear with a cotton swab, and coverslips were mounted with Permount. Cells were counted on a Zeiss microscope with a 40× objective and an 8× eyepiece fitted with a grid scale. Data were expressed as cells per field. One field corresponded to 0.3 mm^2 of the filter area. All experiments were performed at least 3 times in triplicate wells.

Incorporation of [^{3}H]-Thymidine

Incorporation of [^{3}H]-thymidine was measured in growth-arrested BAE cell cultures that were replated at subconfluent density in the presence of [^{3}H]-thymidine (1 μCi/ml) as previously described [Funk and Sage, 1991]. Briefly, cells were replated in 2% serum or, alternatively, on culture dishes that were precoated with 1% gelatin (Fisher Scientific Comp., Fairlawn, NJ) or 5 μg/ml fibronectin (Telios, San Diego, CA) in the absence of FCS. After 1 h, PBS, SPARC, SPARC peptide, and bFGF were added as indicated. After 20 h of incubation, the cells were washed twice with cold PBS, fixed with cold 10% trichloroacetic acid for 10 min, washed with cold ethanol, and air-dried. Subsequently, material insoluble in trichloroacetic acid was hydrolyzed with 200 μl 0.4 M NaOH at 60°C for 5 min, neutralized with 20 μl glacial acetic acid, dissolved in 4 ml Ecolume (ICN, Irvine, CA), and quantified in a scintillation counter.

Plasminogen Activator Assay

Confluent cultures of BAE cells, or cultures that were wounded as described above, were incubated in serum-free DMEM and supplemented with PBS, bFGF, and/or SPARC as indicated. Conditioned medium was collected after 24 h and was clarified by centrifugation (1,200*g* for 10 min). The cells were washed twice with PBS and were extracted with 0.5% Triton X-100 in 0.1 M Tris-HCl buffer (pH 8.1). The amounts of conditioned medium and cell extracts were normalized to cell number, and PA activity was visualized by SDS-PAGE zymography with gelatin and plasminogen copolymerized in the gel, as described by Heussen and Dowdle [1980]. The principal lytic bands were detected at 52 kD and 70 kD, molecular weights that correspond to those of urokinase-type PA (uPA) and tissue-type PA (tPA), respectively [Levin and Loskutoff, 1982]. Identical results were obtained when gelatin was substituted by casein. No activity was detected when plasminogen was omitted from the gel. Incorporation into the gel of anti-bovine uPA IgG (provided by Dr. D.B. Rifkin) neutralized the activity of the M_r 52 kD form, but not the activity of the PA of M_r 70 kD.

Assay for Metalloproteinases

Zymography for metalloproteinases was carried out with culture media and cell extracts as previously described [Herron et al., 1986; Everitt and Sage, 1992] on SDS-gelatin substrate gels. No lytic bands were visible when 10 mM EDTA was added to the incubation buffer.

SPARC-bFGF Binding Studies

To study the interaction of bFGF with SPARC, immunoprecipitations and ligand blotting techniques were performed with [^{125}I]-bFGF (specific activity 50 μCi/μg) (Amersham, Arlington Heights, IL) as previously described for the binding of platelet-derived growth factor (PDGF) to SPARC [Raines et al., 1992]. Alternatively, proteins were transferred to nitrocellulose with a slotblot apparatus (Millipore Corp., Bedford, MA). Nonspecific binding sites on the nitrocellulose were blocked by incubation in blocking solution (PBS containing 1% nonfat dry milk and 0.05% Tween 20, pH 7.4). Slotblots were incubated with 1 μg/ml [^{125}I]-SPARC (specific activity 23.5 μCi/μg). After several washes, the blots were exposed to X-ray film with intensifying screens at −70°C. The density of autoradiographic signals was quantitated with a laser scanning densitometer equipped with an integrator (DU-79 Spectrophotometer, Beckman Instruments).

Determination of High-Affinity Binding of [^{125}I]-bFGF to BAE Cells

The high-affinity binding of [^{125}I]-bFGF to BAE cells was determined as described by Moscatelli and Quarto [1989] with a few modifications. Confluent monolayers of BAE cells were washed twice with cold PBS and were incubated with DMEM containing 0.15% gelatin (pH 7.4), 1 or 10 ng/ml [^{125}I]-bFGF, and PBS, SPARC (40 μg/ml), or bovine serum albumin (BSA) (40 μg/ml). To control for nonspecific binding, parallel cultures were incubated under the same conditions with the addition of a 200-fold excess of unlabeled bFGF. After 2 h of incubation at 4°C, the cells were washed 3 times with PBS and twice with 2 M NaCl in 20 mM HEPES (pH 7.4) to remove [^{125}I]-bFGF bound to low-affinity binding sites. [^{125}I]-bFGF bound to the high-affinity receptors was removed by 2 washes with 2 M NaCl in 20 mM sodium acetate (pH 4.0). The amount of radioactivity released in the washes was quantitated by gamma counter.

RESULTS

The migration of endothelial cells is one of the critical events that mediate wound repair and neovascularization. Previous studies have shown that bFGF stimulates migration of endothelial cells [Gospodarowicz et al., 1987; Sato and Rifkin, 1988]. Since SPARC is associated with cells that are ostensibly participating in tissue remodeling, we examined the role of SPARC in the movement of BAE cells from a wound edge in vitro. After confluent monolayers of BAE cells were scraped with a rubber policeman, PBS, bFGF (10 ng/ml), SPARC (40 μg/ml), or a combination of bFGF and SPARC was added to the wounded cell cultures. Because BAE cells are extremely sensitive to endotoxin in the presence of serum [Hasselaar et al., 1991], and to avoid the possible effects of other (growth) factors, all experiments were conducted in the absence of serum. From serial photographs of the areas marked on the tissue culture dishes, we concluded that BAE cells started moving from the edge of the wound into the denuded area 3 h after scraping (data not shown). Whereas control cultures maintained their characteristic cobblestone morphology during 20 h of incubation (Fig. 1A), addition of bFGF induced an elongated cellular morphology (Fig. 1C). SPARC under these conditions did not affect the morphology of BAE cells (Fig. 1B). Addition of SPARC partially inhibited the morphological change induced by bFGF when SPARC and bFGF were added simultaneously (Fig. 1D). In contrast to experiments with BAE cells that were subconfluent or had just reached confluence [Sage et al., 1989b], SPARC did not cause overt cell rounding. Cells at the wound edge, however, appeared to be less spread in the presence of SPARC. The stabilized interactions that BAE cells in confluent cultures form with a well-established extracellular matrix might protect the cells from the rounding effect of SPARC. In the same wound experiments, the cells that had migrated into the denuded area were counted (Fig. 2A), and the distances that the cells had moved from the wound edge were measured (Fig. 2B). By both criteria, bFGF induced an increase of 1.5- to 2-fold over the migration observed in controls after 20 h. At 40 μg/ml, SPARC significantly ($P < 0.01$) inhibited the increase in the number of migrated cells, as well as the increase in the distance of migration, that had been observed in the presence of bFGF.

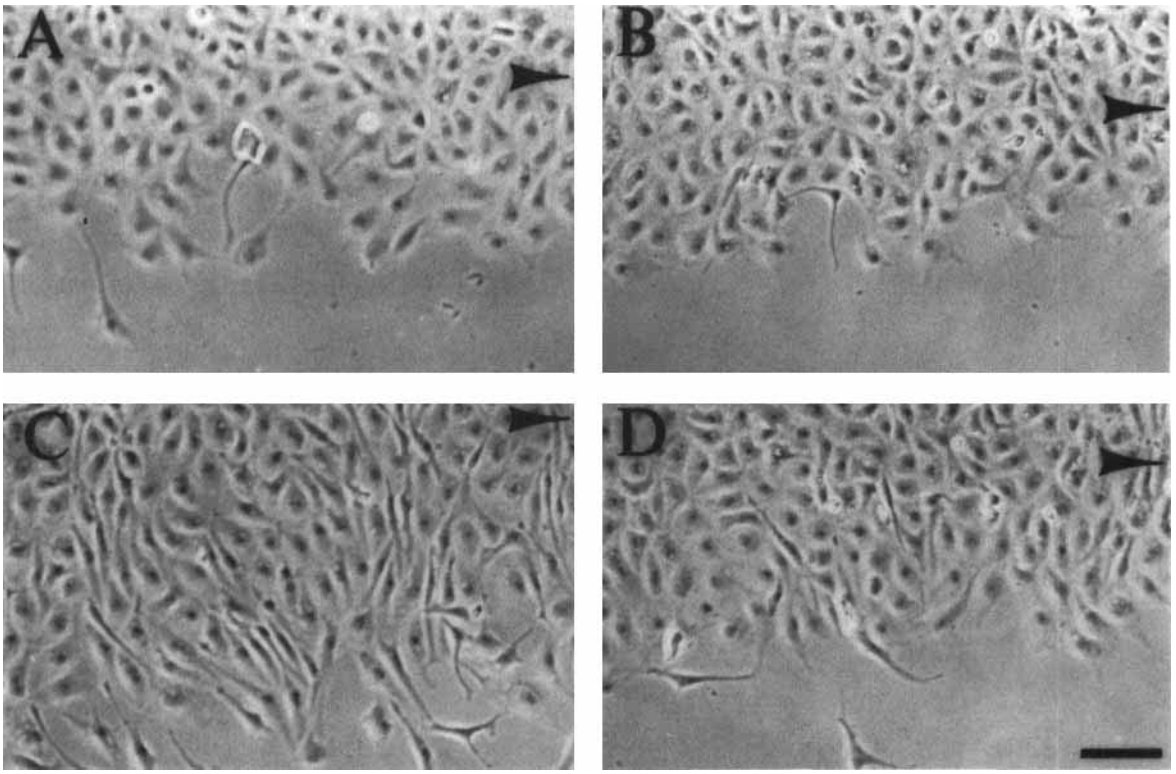

Fig. 1. Effect of bFGF and SPARC on the migration of BAE cells. Confluent monolayers of BAE cells were wounded with a cell scraper as described in Materials and Methods. The cells were incubated for 20 h in the indicated media and photographed. **A:** Control. **B:** SPARC (40 μg/ml). **C:** bFGF (10 ng/ml). **D:** SPARC (40 μg/ml) and bFGF (10 ng/ml). The arrowheads were placed at the original edges of the wounds. Bar, 50 μm.

SPARC did not have an effect in the absence of bFGF.

To confirm our observation that SPARC modulates the effect of bFGF on the movement of BAE cells, we performed a series of migration experiments with a Boyden chamber. Except for the experiments described in Table I, all Boyden chamber assays were conducted in the absence of serum. Initially, the directed migration of BAE cells in response to different concentrations of bFGF (0.03–10 ng/ml) was investigated (Fig. 3A). Total migration was stimulated significantly by bFGF and exhibited a maximal increase of 1.5-fold over control values with 0.3 ng/ml of bFGF. In addition, bFGF was found to be chemokinetic for BAE cells, since a similar response was elicited by bFGF upon elimination of the bFGF concentration gradient. To determine the effect of SPARC in this system, different concentrations of SPARC were added to the lower wells of the Boyden chamber, either in the absence or presence of 0.3 ng/ml bFGF (Fig. 3B). As we had observed in the wound assay, SPARC alone had no effect on the motility of BAE cells, but it inhibited the migration induced by bFGF. This effect was dependent on the concentration of SPARC, with a complete inhibition to control values at 60 μg/ml; preparations of bovine and mouse SPARC were equally effective. Since it is known that SPARC binds to BSA [Sage et al., 1984] and that low levels of contaminating endotoxin are present in preparations of SPARC [Hasselaar et al., 1991], we tested the effects of both BSA and endotoxin on the migration of BAE cells induced by bFGF. Neither BSA nor endotoxin had an inhibitory effect on cell migration. The observed effects on BAE cell migration therefore appeared to result directly from exposure of the cells to SPARC. In contrast to the effect of SPARC on migration in the absence of serum, 60 μg/ml SPARC did not inhibit BAE cell motility induced by bFGF in the presence of 1% FCS in the Boyden chamber assay (Table I).

Different synthetic peptides representing subregions of SPARC were used in the Boyden chamber to determine sequence motifs that might be responsible for the observed inhibitory effect. As shown in Figure 4, peptides from two Ca^{2+}-binding domains (peptides 1.1 and 4.2)

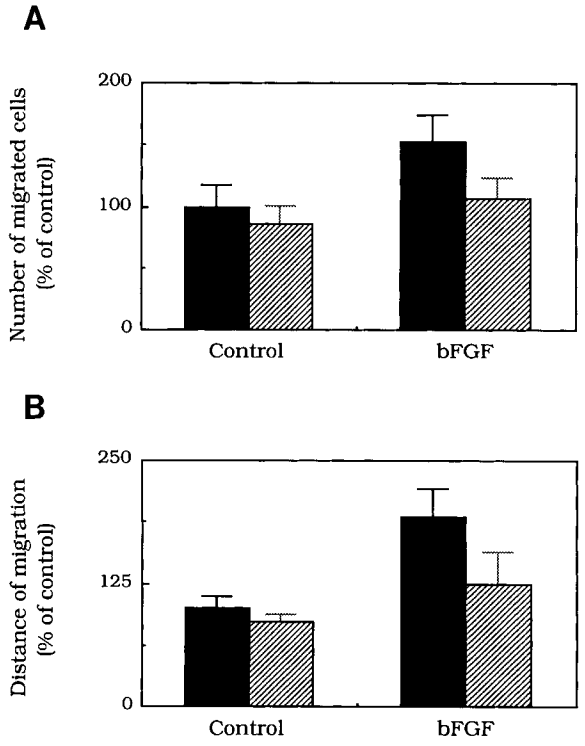

Fig. 2. SPARC inhibits the migration of BAE cells induced by bFGF in wound assays in vitro. Confluent monolayers of BAE cells were wounded with a cell scraper and subsequently incubated for 20 h with PBS (control) or bFGF (10 ng/ml) in the absence (closed bars) or presence (hatched bars) of SPARC (40 μg/ml). The number of cells that had migrated from the wound edge was counted (**A**), and the distance of cell migration was measured (**B**). Results were expressed as percentage of control (mean ± SD).

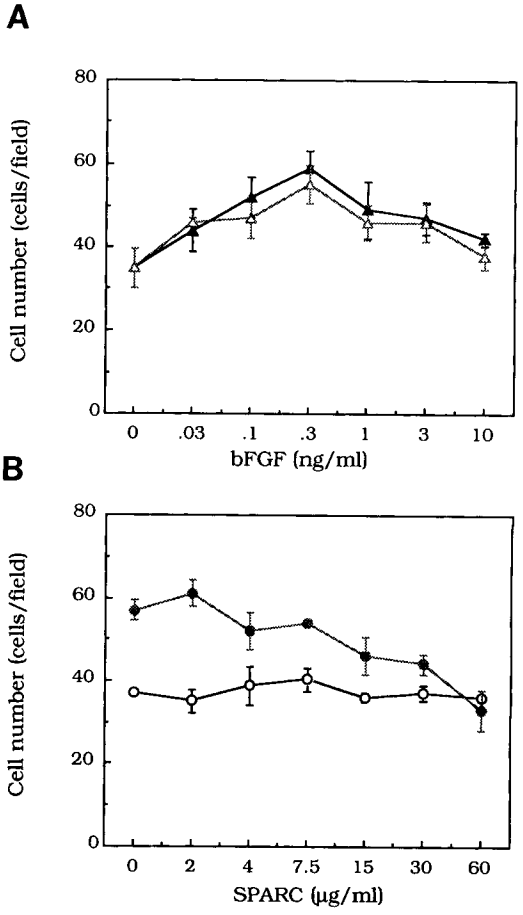

Fig. 3. SPARC inhibits the migration of BAE cells induced by bFGF in a Boyden chamber assay. Experiments were performed with BAE cells as described in Materials and Methods. **A:** Different concentrations of bFGF were added to the lower wells to measure total cell migration (closed triangles), or to both upper and lower wells to measure chemokinesis (open triangles). **B:** The migratory response of BAE cells to different concentrations of SPARC, in the absence (open circles) or presence (closed circles) of 0.3 ng/ml bFGF, was determined. SPARC and bFGF were added to the bottom wells of the Boyden chambers. Results were expressed as the number of migrated cells per field (mean ± SD).

mimicked the effect of intact SPARC and showed a comparable inhibition of cell migration in response to bFGF in serum-free medium. Peptides from a cysteine-rich domain (peptide 2.1) and an extended α-helical domain (peptide 3.4) did not have inhibitory effects. These findings indicate provisionally that the inhibition is Ca^{2+} dependent. To examine whether SPARC and peptide 4.2 required Ca^{2+} for their effects on cell migration, we performed Boyden chamber experiments in the presence of either EGTA or $CaCl_2$ (Table II). Addition of 1 mM $CaCl_2$ did not affect the motility of BAE cells, and 2 mM EGTA did not inhibit the migratory response of BAE cells to bFGF. However, incubation of SPARC or peptide 4.2 with EGTA diminished the ability of the protein or peptide to inhibit the migration induced by bFGF. Moreover, a mutant peptide 4.2 that should not bind Ca^{2+} (substitution of D into K at position 258) inhibited cell migration in response to bFGF by 50%. Addition of equimolar concentrations of the Ca^{2+}-binding protein

TABLE I. SPARC Does Not Inhibit BAE Cell Migration in Response to bFGF in the Presence of Serum*

Additions	PBS	SPARC
PBS	41.4 ± 8.8	49.0 ± 6.5
bFGF	65.2 ± 11.9	66.7 ± 17.3

*Boyden chamber assays were performed in the presence of 1% FCS. Under these conditions, 3 ng/ml bFGF was required to induce a 1.5-fold stimulation of BAE cell migration (not shown). PBS, SPARC (60 μg/ml), and bFGF (3 ng/ml) were incubated in the lower wells of the Boyden chamber. Both the upper and lower wells of the chambers contained 1% FCS. Results were expressed as the number of migrated cells per field (mean ± SD).

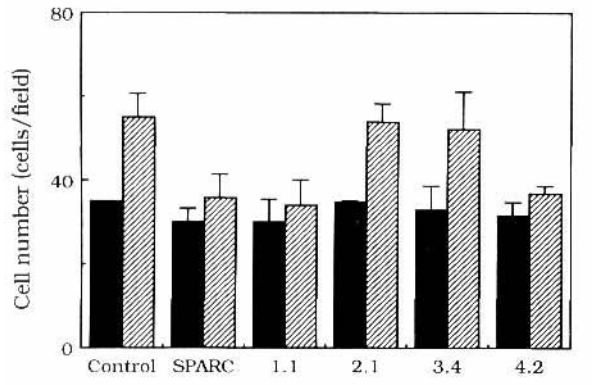

Fig. 4. N- and C-terminal SPARC peptides mimic the inhibitory effect of SPARC on the migration of BAE cells induced by bFGF. Experiments were performed as described in Materials and Methods. SPARC (60 μg/ml) and SPARC peptides 1.1, 2.1, 3.4, and 4.2 (0.4 mM) were incubated in the absence (closed bars) or presence (hatched bars) of 0.3 ng/ml bFGF in the bottom wells of the Boyden chamber. Results were expressed as the number of migrated cells per field (mean ± SD).

TABLE II. Effects of Ca^{2+} and EGTA on the Migration of BAE Cells Induced by bFGF in a Boyden Chamber Assay*

Additions	PBS	Ca^{2+}	EGTA
bFGF	55.0 ± 5.6	51.3 ± 7.0	53.1 ± 6.5
bFGF/SPARC	35.8 ± 5.8	40.2 ± 4.0	48.4 ± 7.2
bFGF/peptide 4.2	36.7 ± 2.0	33.9 ± 4.0	52.2 ± 2.3

*Combinations of bFGF (0.3 ng/ml), SPARC (60 μg/ml), peptide 4.2 (0.4 mM), $CaCl_2$ (1 mM), and EGTA (2 mM) were prepared in the combinations shown and were added to the bottom wells of the Boyden chamber. The effects of the various reagents on the migration of BAE cells induced by bFGF were expressed as the number of cells per field (mean ± SD). Control migration (no additions) was 35 cells/field.

calmodulin, relative to SPARC, did not affect the motility of BAE cells, either in the absence or presence of bFGF. Finally, heating of peptide 4.2 for 5 min at 100°C partially eliminated (50% inhibition) the abilily of peptide 4.2 to antagonize migration induced by bFGF.

Because SPARC has previously been shown to inhibit cell cycle progression in BAE cells [Funk and Sage, 1991], and since bFGF is a well-known stimulator of endothelial cell proliferation [Moscatelli et al., 1986; Presta et al., 1986; Isacchi et al., 1991], we analyzed the effect of SPARC on the mitogenic response of BAE cells to bFGF. In the presence of 2% FCS and with all concentrations of SPARC tested, 5 ng/ml bFGF induced a twofold increase in the incorporation of [^{3}H]-thymidine into newly synthesized DNA (Fig. 5A). Both in the absence and presence of bFGF, SPARC inhibited [^{3}H]-thymidine incorporation in a dose-dependent manner. When DNA synthesis was plotted as percentage of control (incorporation of [^{3}H]-thymidine by BAE cell cultures that did not receive exogenous SPARC), the percentage of inhibition by different concentrations of SPARC was the same in both the absence and presence of bFGF (Fig. 5A, insert). This finding indicates that SPARC elicits growth-

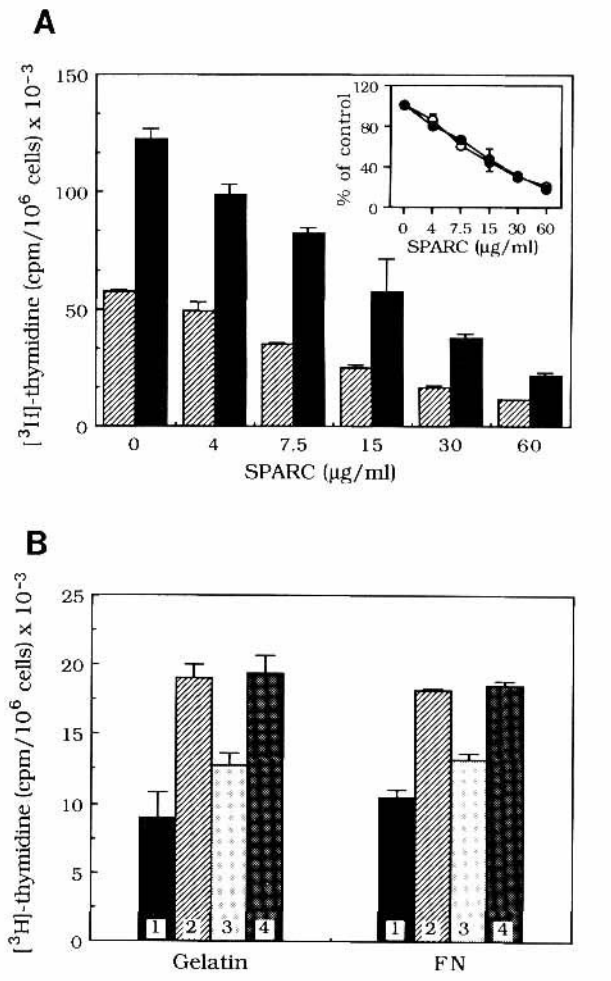

Fig. 5. SPARC inhibits DNA synthesis in the presence of serum, but not in the absence of serum. Incorporation of [^{3}H]-thymidine was measured in BAE cell cultures as described. **A:** Dose-response curve of SPARC (0–60 μg/ml), in the absence (hatched bars) or presence (closed bars) of 5 ng/ml bFGF, in medium containing 2% FCS. **Insert:** The same data were expressed as percentage of [^{3}H]-thymidine incorporation of control (the level of incorporation in the absence of exogenous SPARC was 100%), either in the absence (open circles) or presence (closed circles) of bFGF. **B:** DNA synthesis by BAE cells in serum-free medium, on substrates of gelatin and fibronectin (FN), after the addition of PBS (bar 1), 5 ng/ml bFGF (bar 2), 30 μg/ml SPARC (bar 3), and bFGF + SPARC (bar 4). Results were expressed as the amount of [^{3}H]-thymidine (cpm) that was incorporated into the DNA of 10^6 BAE cells in 20 h (mean ± SD).

arrest of a defined number of cells, regardless of bFGF. Cells that are unaffected by SPARC could still respond to the growth-stimulatory effect of bFGF. Peptide 4.2, added at the concentration used in the migration experiments (0.4 mM), had no effect on the incorporation of [^{3}H]-thymidine in the absence or presence of bFGF. When BAE cells were plated on substrates of gelatin or fibronectin in the absence of serum, the incorporation of [^{3}H]-thymidine was also stimulated twofold with 5 ng/ml bFGF (Fig. 5B). Unlike the inhibitory effect on DNA synthesis that was seen with SPARC in the presence of serum (70% inhibition with 30 μg/ml SPARC; Fig. 5A), 30 μg/ml SPARC stimulated DNA synthesis to a slight extent in the absence of serum (Fig. 5B, bar 3). In addition, SPARC had no effect on the mitogenic effect of bFGF under these conditions (Fig. 5B, bar 4). When the assay was performed on fibronectin-coated dishes in the presence of 2% FCS, however, SPARC and bFGF affected DNA synthesis as described in Figure 5A for serum-containing cultures. In addition, SPARC and bFGF elicited the same respective inhibitory and stimulatory responses when FCS was substituted by plasma-derived serum. This result suggests that the inhibitory effect of SPARC on DNA synthesis is mediated by a serum factor that is not released by blood platelets.

Several studies suggest that the expression of uPA is involved in the migration of several types of cells such as human and bovine endothelial cells and keratinocytes [Pepper et al., 1987; Morioka et al., 1987; Sato and Rifkin, 1988; Mawatari et al., 1991]. Because PAI-1, the primary physiologic inhibitor of both uPA and tPA, is induced by SPARC in BAE cells [Hasselaar et al., 1991], we asked whether SPARC would modulate the migration of BAE cells indirectly through inhibition of the activity of cell-associated PA. Confluent and wounded cultures of BAE cells were incubated in serum-free DMEM in the absence or presence of SPARC (40 μg/ml) and bFGF (10 ng/ml). After 24 h, conditioned medium and cell extracts were collected and analyzed for PA activity (Fig. 6A). Cell-associated and secreted levels of active uPA (and of

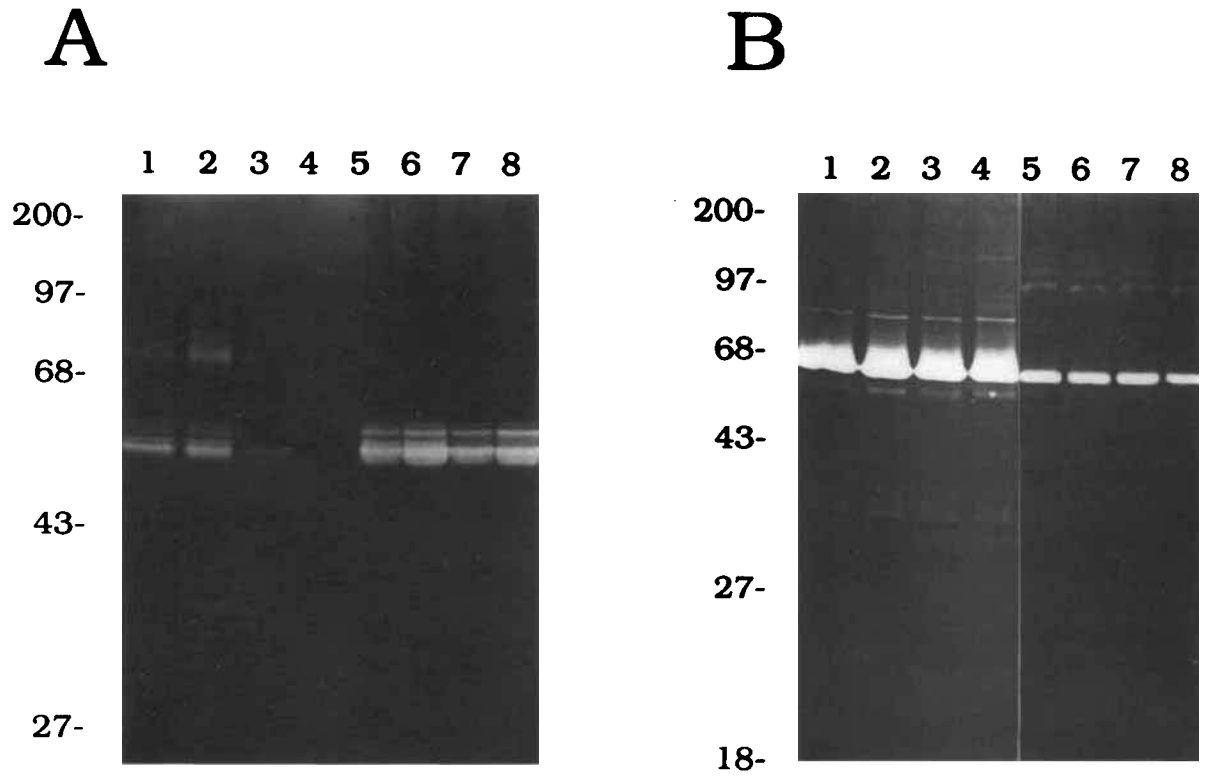

Fig. 6. Zymography for PA and metalloproteinase activity. BAE cells were incubated for 24 h with PBS (lanes 1 and 5), 10 ng/ml bFGF (lanes 2 and 6), 50 μg/ml SPARC (lanes 3 and 7), or both bFGF and SPARC (lanes 4 and 8). Conditioned medium (lanes 1–4) and cell extracts (lanes 5–8) were analyzed for PA activity (**A**) and metalloproteinase activity (**B**). Positions of molecular weight standards are indicated ($M_r \times 10^{-3}$).

secreted, active tPA) were slightly elevated after the addition of bFGF (Fig. 6A, lanes 2 and 6). Addition of SPARC did not alter the basal or bFGF-induced expression of cell-associated uPA (lanes 7 and 8), but it decreased the secretion of both PAs into the medium (lanes 3 and 4). In addition, cells that were incubated for 24 h with 0.4 mM peptide 4.2 expressed increased levels of cell-associated uPA and decreased levels of secreted uPA, either in the presence or absence of bFGF (not shown). The same zymography results were obtained with confluent and wounded BAE cells (not shown). Subsequently, the samples from Fig. 6A were analyzed for metalloproteinase activity (Fig. 6B). Areas of lysis at M_r 85 kD, 60 kD, 55 kD, and 36 kD were observed when proteinases secreted by BAE cells were analyzed on gelatin substrate gels. The molecular weights of two of the lytic bands are highly similar to those of previously reported metalloproteinases secreted by bovine capillary endothelial cells [Tsuboi et al., 1990] and rabbit brain capillary endothelial cells [Herron et al., 1986]. In cell extracts, metalloproteinases of 90 kD and 60 kD were seen. In both cell extracts and conditioned medium, no significant differences in the patterns of expression of metalloproteinases or in their levels of activity were found when BAE cells were incubated with PBS, bFGF, and/or SPARC. Also, no significant differences in expression were found between confluent and wounded cell cultures (not shown).

Because SPARC binds to PDGF-AB and PDGF-BB [Raines et al., 1992], we performed binding experiments to determine whether the effect of SPARC on bFGF-induced cell migration was the result of a molecular interaction between SPARC and bFGF. No specific binding of [^{125}I]-bFGF to SPARC was observed in either ligand blot or solution immunoprecipitation assays (data not shown). By slotblot analysis, we also failed to demonstrate an interaction between [^{125}I]-SPARC and bFGF (500 ng), whereas the same preparation of [^{125}I]-SPARC bound to type III collagen (5 μg) and PDGF-AB (50 ng) (Fig. 7).

Finally, receptor binding studies were performed to test whether SPARC might interfere with the high-affinity binding of bFGF to BAE cells. As shown in Figure 8, neither SPARC nor BSA inhibited the specific binding of 1 ng/ml or 10 ng/ml [^{125}I]-bFGF to its cognate receptors on BAE cells.

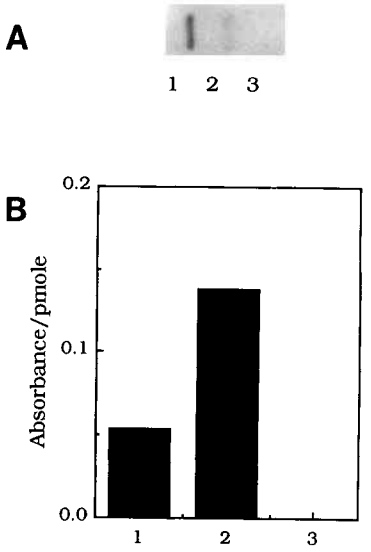

Fig. 7. SPARC does not bind to bFGF. Five micrograms (24 pmoles) type III collagen (slot 1, lane 1), 50 ng (1.6 pmoles) PDGF-AB (slot 2, lane 2), and 500 ng (28 pmoles) bFGF (slot 3, lane 3) were transferred to nitrocellulose with a slotblot apparatus and were subsequently incubated with [^{125}I]-SPARC. After several washes, the blot was exposed to X-ray film (**A**), and images corresponding to the radioactive bands were quantitated by densitometry and expressed as absorbance/pmole (**B**).

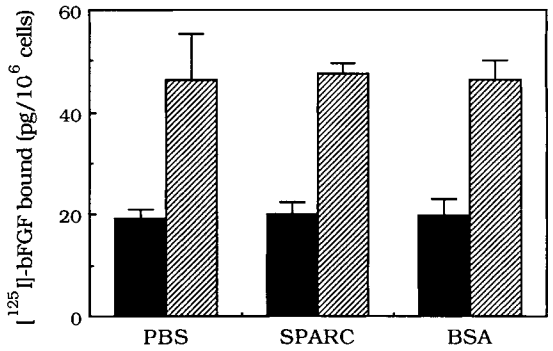

Fig. 8. SPARC does not perturb the binding of [^{125}I]-bFGF to high-affinity receptors. Confluent cultures of BAE cells were incubated with 1 ng/ml (closed bars) or 10 ng/ml (hatched bars) of [^{125}I]-bFGF and PBS, 40 μg/ml SPARC, or 40 μg/ml BSA. Nonspecific binding was determined by incubation of parallel cultures with the above media containing a 200-fold excess of unlabeled bFGF. Results are expressed as specific high-affinity binding of bFGF to receptors on BAE cells (pg/10^6 cells) (mean ± SD).

DISCUSSION

The morphogen bFGF plays an important role in the formation of new blood vessels (neovascularization or angiogenesis), a process that is characterized by penetration of endothelial cells into the surrounding tissues, migration of the

cells toward a source of angiogenic factor, and endothelial cell proliferation. Although the regulation of this complex process is still unclear, it is generally believed that proteolytic degradation of components of the extracellular matrix is required. Angiogenic stimuli (e.g., bFGF) induce the expression of proteinases such as PAs and collagenases [Presta et al., 1986; Moscatelli et al., 1986; Montesano et al., 1986; Sato and Rifkin, 1988]. PAs convert plasminogen into plasmin, a serine protease that can degrade several extracellular matrix molecules that include fibrin, fibronectin, and laminin [Saksela, 1985; Liotta et al., 1981]. Plasmin also converts procollagenase into its active form [Werb et al., 1977]. A high-affinity receptor for uPA on various cell types restricts uPA to migrating cells [Pepper et al., 1987; McNeill and Jensen, 1990; Estreicher et al., 1990] and effectively limits the activity of the PA/plasmin system to the immediate cellular environment.

The extracellular Ca^{2+}-binding glycoprotein SPARC is also associated with remodeling, cellular migration, and proliferation [Mason et al., 1986a,b; Holland et al., 1987; Sage et al., 1989a,b]. Moreover, synthesis of SPARC is reinitiated in sprouting endothelial cells that form tubular structures in vitro [Iruela-Arispe et al., 1991]. Addition of SPARC to BAE cells in culture elicits a rounded morphology that is correlated with an inhibition of cell spreading [Sage et al., 1989b]. SPARC also inhibits progression of the BAE cell cycle from G_1 to S phase [Funk and Sage, 1991] and induces the expression of PAI-1 in subconfluent cultures of these cells [Hasselaar et al., 1991]. These findings indicate that SPARC might be a regulatory component of the invasive and migratory aspects of angiogenesis.

The results from this study demonstrate that, in the absence of serum, SPARC antagonizes the stimulatory effect of bFGF on the migration of BAE cells in both an in vitro wound assay and a Boyden chamber assay. However, the basal motility of BAE cells was not affected by SPARC. Since mechanical injury such as scraping of endothelial cells results in the rapid release (within 5 min) of bFGF-like activity into the culture medium [McNeil et all., 1989], one might predict that SPARC should have inhibited cell migration in the absence of exogenous bFGF. Extensive washing of the cell cultures after scraping *most likely* eliminated the majority of the bFGF activity. Moreover, low levels of endogenous bFGF released from the injured cells could have bound instantaneously to the extracellular matrix [Vlodavsky et al., 1987] and would thus be unavailable.

In analogy to our findings with SPARC and bFGF, Mawatari et al. [1991] showed that tumor necrosis factor inhibited the stimulatory effect of epidermal growth factor on the migration of human microvascular endothelial cells. The production of PAI-1 was concomitantly increased, and that of tPA decreased, in these arrested cultures. Since bFGF stimulates the expression of PA in BAE cells [Sato and Rifkin, 1988; Isacchi et al., 1991], and SPARC, in contrast, induces the synthesis of PAI-1 [Hasselaar et al., 1991], we questioned whether SPARC suppressed the cell migration induced by bFGF through an inhibition of PA activity. As shown by zymography, both cell-associated and secreted levels of active PA were slightly increased by exogenous bFGF. Exogenous SPARC significantly inhibited the activity of secreted PAs, both in the presence and absence of bFGF, but it did not inhibit the expression of cell-associated active uPA. If SPARC antagonizes endothelial cell migration by diminishing the levels of secreted uPA, it would be expected that SPARC not only inhibit the migration of BAE cells in response to bFGF, but also the basal cell motility. Furthermore, since cell-associated levels of uPA are unaffected by SPARC and even increased by peptide 4.2, it is unlikely that modulation of the cell-associated PA/plasmin system is responsible for the effect of SPARC on cell migration. In fact, in a recent study with clones of bovine capillary endothelial cells, Tsuboi et al. [1990] were unable to demonstrate a correlation between the extent of cellular migration and the activity of cell-associated PA. Their zymography data, however, indicate that the levels of metalloproteinases secreted into the conditioned medium might be important for cell migration and invasion. We therefore analyzed BAE cell extracts and culture media for metalloproteinase activity. As shown in Figure 6B, no significant differences in cell-associated or secreted levels of metalloproteinases were found. This finding is in agreement with the zymography data from stably transfected F9 embryonal carcinoma cell lines that over- or underproduce SPARC protein [Everitt and Sage, 1992]. In these cells, there was no variation from control levels with respect

to secreted or cell-associated metalloproteinases. In our study, BAE cell migration did not appear to correlate with the induction/activation of either specific metalloproteinases or the PA/plasmin system. These proteolytic systems might therefore not be functional or even necessary for cell migration as measured by wounding and Boyden chamber assays. However, relevant differences in protease expression at the leading edge of the migrating cell sheet cannot be excluded at this time.

The modulatory effect of SPARC on the responses of BAE cells mediated by bFGF in the absence of serum appeared to be restricted to cell migration, because stimulation of DNA synthesis by bFGF was unaffected by SPARC under these conditions. However, as we reported previously, SPARC inhibited DNA synthesis in the presence of serum [Funk and Sage, 1991], as well as in the presence of plasma-derived serum. These data indicate that the inhibitory effect of SPARC on cell proliferation is mediated by components present in plasma and not by factors that are released by blood platelets. Inhibition of cell proliferation by SPARC could conceivably occur through a molecular interaction between SPARC and the putative factor(s). The mechanism by which SPARC exerts its effect on cell-cycle progression is currently under investigation. It is interesting to note that in the presence of serum, when DNA synthesis is inhibited by SPARC, endothelial cell migration is unaffected. In contrast, in the absence of serum, the cells can proliferate and migration is inhibited by SPARC.

We have recently shown that SPARC interacts with PDGF-AB and PDGF-BB, but not with PDGF-AA [Raines et al., 1992]. In a number of conditions associated with injury and remodeling, SPARC could therefore limit the availability of PDGF and consequently control directed cell migration and proliferation. These findings suggested to us that the effect of SPARC on bFGF-induced cell migration could be explained by an interaction between SPARC and bFGF. Using different preparations of SPARC and bFGF in conjunction with a variety of binding assays, we were unable to detect a specific interaction between the two molecules. In addition, SPARC did not appear to interfere with the high-affinity binding of bFGF to its plasma membrane receptor. Isacchi and coworkers [1991] have suggested that the PA-inducing activity of bFGF depends on a functional domain that differs from those that contribute to the mitogenic activity of bFGF. Moreover, two different signal transduction pathways have been shown to mediate PA induction and mitosis in endothelial cells [Presta et al., 1989]. That only the migratory response to bFGF is blocked by SPARC, but not the induction of cell-associated PA activity and DNA synthesis (in the absence of serum), could also be explained by the presence of different functional domains within bFGF. In addition, SPARC might specifically modulate the signal transduction pathway that functions during the induction of migration by bFGF.

Although we have not identified a precise mechanism by which SPARC exerts its effect on cell migration, we have provided evidence that the two Ca^{2+}-binding domains of SPARC (peptides 1.1 and 4.2) are involved in this activity. Because chelation of Ca^{2+} with EGTA diminished the antagonizing effect of SPARC and peptide 4.2 on cell migration induced by bFGF, and since heating partially eliminated the inhibitory effect of peptide 4.2, it appears that Ca^{2+} is required for the maintenance of the appropriate conformation of SPARC. Finally, reorganization of actin filaments in BAE cells after the addition of SPARC (J.C. Yost and E.H. Sage, unpublished observations) could explain the altered morphology of the cells and the inability to mobilize in response to a migratory stimulus.

In summary, we propose that a high local concentration of SPARC, expected to be present at sites of vascular injury or during angiogenesis, will not only control the proliferation of endothelial cells, but also regulate the migratory response of endothelial cells to bFGF. SPARC might therefore provide the negative feedback necessary to control highly proliferative and migratory responses in remodeling tissues. Because migration is inhibited by SPARC under serum-free conditions when DNA synthesis is not affected by SPARC and, in contrast, cells are allowed to migrate normally in the presence of serum when SPARC inhibits endothelial cell proliferation, we propose that SPARC, in concert with plasma-derived factors, regulates the switch between growth and migration of endothelial cells.

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