SPARC is a VCAM-1 counter-ligand that mediates leukocyte transmigration

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Abstract: VCAM-1 is a cell surface molecule, which has been shown to mediate leukocyte adhesion to the endothelium and subsequent transmigration. Although VCAM-1 regulates adhesion through its interaction with VLA-4, VLA-4 does not play a role in VCAM-1-dependent diapedesis, an observation suggesting the presence of a second ligand for VCAM-1. We now report a novel interaction between VCAM-1 and secreted protein acidic and rich in cysteine (SPARC), which induces actin cytoskeletal rearrangement and intercellular gaps, physiological processes known to be important for leukocyte transmigration. The binding of leukocyte-derived SPARC to VCAM-1 was demonstrated to be necessary for leukocyte transmigration through endothelial monolayers (diapedesis) in vitro, and furthermore, SPARC null mice have abnormalities in leukocyte recruitment to the inflamed peritoneum in vivo. These findings provide new insight into the mechanisms of transendothelial leukocyte migration and suggest a potential, targetable interaction for therapeutic intervention. J. Leukoc. Biol. 81: 000–000; 2007.

Key Words: adhesion molecules • cell trafficking

INTRODUCTION

Migration of circulating leukocytes from the vasculature into the surrounding tissue (leukocyte extravasation) is an important component of the inflammatory response. Leukocyte extravasation is a multistep process in which leukocytes first adhere loosely to and roll on the vascular endothelium, after which they adhere strongly, arrest, and spread on the apical surface and finally, traverse the vascular endothelium and basement membrane (diapedesis) [1]. Each stage comprises a sequence of physical interactions and signaling events mediated by specific cell surface molecules on the leukocyte and the endothelium. Of the steps that mediate leukocyte extravasation, diapedesis remains the least-characterized. Despite the identification of a number of inter- and intracellular events necessary for leukocyte/endothelial transmigration, a complete and definitive account of the sequence of events mediating diapedesis remains to be elucidated [2].

Among the cell surface molecules identified as contributing to leukocyte extravasation is VCAM-1 (CD106), an Ig-like glycoprotein of 100–110 kDa, which is expressed on activated vascular endothelium [3] and is important in atherogenesis [4] and other inflammatory pathologies [5]. VCAM-1 participates in all stages of leukocyte-endothelial interaction. It contributes to rolling and firm adhesion via binding to its cognate ligand, the integrin VLA-4, expressed on the surface of leukocytes [6]. However, not all VCAM-1-mediated events can be linked to VLA-4. It is notable that subsequent to firm adhesion of leukocytes to vascular endothelial cells, VCAM-1-mediated signaling initiates cytoskeletal remodeling [7]. Although it is known that signaling mediated by leukocyte adhesion to endothelial VCAM-1 is required for diapedesis, the identity of the leukocyte surface ligand mediating this signaling has not been demonstrated conclusively. Furthermore, that leukocyte integrins have been shown to play no role in diapedesis eliminates the possibility that VLA-4 is responsible for more than adhesion [8]. The existence of such gaps in the current models of diapedesis, despite intensive study of the molecules known to be involved, suggests the participation of as-yet unidentified cell surface molecule(s) in leukocyte transendothelial migration.

To identify novel contributors to VCAM-1-mediated pathogenesis, we used a phage-display approach that allowed us to screen a diverse array of biomolecules with no a priori biases. Our screen identified a cluster of peptides similar in sequence to the 32-kDa matricellular glycoprotein secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or BM-40. The possibility that SPARC binds to VCAM-1 is intriguing, given the similarity of the sets of events that each protein is known to regulate. SPARC is known to bind endothelial cells [9] and like VCAM-1, mediates cytoskeletal rearrangement and vascular permeability [10–14], although the identification of a SPARC cell surface receptor responsible for this function has not been reported previously. In this report, we demonstrate a novel role for VCAM-1 as a cell surface
receptor for SPARC and present evidence that interaction between VCAM-1 and leukocyte-derived SPARC is necessary for diapedesis, actin cytoskeletal rearrangement, and the appearance of intercellular gaps. SPARC also compromises endothelial barrier function over a time-scale relevant to leukocyte transmigration. We demonstrate that SPARC binds to VCAM-1 in a specific, concentration-dependent manner and that incubation of vascular endothelium with exogenous SPARC under flow conditions has no effect on leukocyte adhesion but significantly inhibits subsequent transendothelial migration. Furthermore, we demonstrate that leukocyte-derived SPARC is necessary for diapedesis in vitro and that SPARC plays an important role in leukocyte recruitment in vivo. This newly described interaction provides a point of intervention relevant to inflammatory pathologies.

MATERIALS AND METHODS

All materials were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Fisher unless otherwise noted.

Antibodies

All antibodies were purchased commercially as follows: goat anti-human (anti-h)SPARC was obtained from R&D Systems (Minneapolis, MN, USA); bovine antigo IgG-HRP conjugate was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-M13-biotin conjugate was obtained from ExAlpha Biologicals (Boston, MA, USA); anti-rabbit IgG Texas Red conjugate was obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA); anti-M13 rabbit IgG conjugate was from AbCam (Cambridge, MA, USA); antirabbit IgG Texas Red conjugate was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); mouse anti-hVCAM-1-CD106 [Clone BBIG-VI(4|2)] and mouse anti-ICAM-1/ICD54 [Clone BBIGII-11|IGBl] were from R&D Systems.

Mice

Wild-type (C57Bl1/6), female mice, ages 7–8 weeks, were obtained from Taconic Laboratories (Germantown, NY, USA) and were used in static and flow transmigration experiments. C57BL/6 × 129SVJ SPARC null mice were generated as described in Norose et al. [15]. Mice were maintained in approved institutional housing and were killed by CO2 asphyxiation as approved by the Panel on Euthanasia of the American Veterinary Association.

Cells/cell culture: isolation of primary leukocytes

Primary human PMN and mononuclear cells (MNC) were isolated as described previously [16].

Isolation and culture of endothelial cells

HUVEC were isolated from two to five umbilical cord veins, pooled, and established as primary cultures in M199 containing 20% (v/v) FCS as described previously [17]. HUVEC cultures were serially passaged and maintained in M199 containing 10% FCS, endothelial cell growth factor, heparin, and antibiotics as described [17]. Murine cardiac endothelial cells (MCEC) and murine lung endothelial cells (MLEC) were isolated as reported previously [18]. Briefly, hearts or lungs of three mice were harvested, minced finely, and digested in 25 ml collagenase (Worthington Biochemical, Lakewood, NJ, USA; 2 mg/ml). Endothelial cells were isolated magnetically with anti-PECAM-1-coated beads (Dynal, Great Neck, NY, USA), washed with complete media and murine lung endothelial cells (MLEC) were isolated as reported previously [17]. Murine cardiac endothelial cells (MCEC) were established as primary cultures in M199 containing 20% (v/v) FCS as described in Norose et al. [15]. HUVEC cultures were serially passaged and maintained as described previously [16]. Briefly, MCEC were plated onto 25 mm diameter coverslips ( precoated with 1 µg/cm² hFN) and subsequently placed in the flow chamber at 37°C. Phage (10¹⁰ PFU), displaying random disulfide-constrained seven amino acid peptides (G7C-PhD, New England Biolabs, Beverly, MA, USA), were drawn across the monolayers for 10 min at 0.32 ml/min (estimated wall shear stress of 1.0 dynes/cm²). Coverslips were removed, placed into complete media, and incubated at 37°C for an additional 15 min. The bound extracellular (EC)-restricted phage was removed with 0.2 mol/L glycine, pH 2.2 (3X5 min). Internalized phage was recovered by lysis of MCEC with 0.1% triethylammonium hydroxide in PBS, pH 7.4 (4 min, room temperature). Both extracts were neutralized with 500 µl 0.5 mol/L Tris-HCL (pH 9.0) and were quantified by a plaque-forming assay. For negative selection, MLEC were incubated for 30 min at 37°C in complete media with the dialyzed phage from the first round of positive selection. Unbound phage was then transferred to a new monolayer of MLEC, and the procedure was repeated for a total of three rounds of negative selection. Phage was amplified by Escherichia coli, titrated, and used for three additional rounds of positive selection. Phage recovered from the fourth round was plated, and individual clones were selected for ELISA assay. Clones (n=8), with high affinity, as determined by ELISA, were sequenced.

ELISA of phage clone-binding to VCAM-1

The capacity of selected phage clones to bind to purified VCAM-1 was determined by ELISA as described previously [19]. Briefly, protein A (20 µg/ml) was spotted onto flat-bottom Nunc Maxisorp plates overnight at 4°C. Plates were incubated sequentially at room temperature with soluble Fc-VCAM-1 (10 µg/ml, 1 h) and phage clones (10¹⁰ PFU, 1 h), washed with PBS containing 0.1% Tween-20, and incubated with biotinylated αM13 antibody (1:40) for 1 h. Detection was performed with streptavidin-HRP (1:500), followed by color development using an ELISA reader (Emax, Molecular Devices, Sunnyvale, CA, USA). Relative absorbance was calculated by subtracting the average of background wells (n=20) from each data point and dividing by the absorbance of the highest binding clone.

Immunohistochemistry

MCEC were plated on fibronectin-coated chamber slides and incubated with 10¹⁰ PFU M9KSHIPAC peptide displaying phage, with or without preincubation with 5× murine (m)VCAM-1/Fc or mVCAM-1/Fc chimeric protein (R&D Systems). Cells were washed 3X with PBS containing 0.1% (v/v) tween-20, incubated with rabbit anti-M13 (1:500 dilution), followed by antirabbit IgG-Texas Red (1:1000 dilution), and were visualized by fluorescence microscopy on a Nikon Eclipse TE2000-S, Insight QE, 20X objective.

Coprecipitation of SPARC and FcVCAM-1

All protein-binding reactions were incubated for 1 h at 37°C. Recombinant (r)VCAM-1/Fc chimeric protein (1 µg) was incubated with 1 µg or 10 µg SPARC, purified from human leukocytes (Haematologic Technologies, Essex Junction, VT, USA). As controls, 1 µg rhCAM-1/Fc chimeric protein was also incubated with 10 µg SPARC, and 10 µg SPARC was incubated with vehicle alone (PBS).

Protein G-sepharose beads (30 µl), prewashed with PBS, were blocked to prevent nonspecific protein binding by resuspension in 30 µl Dulbecco’s PBS (DPBS)+/−10 mM Mg²⁺/1 mM Ca²⁺ (Cambrex Bio Science, Baltimore, MD, USA) containing 1.0% (w/v) BSA for 1 h at room temperature with rocking. The suspension was aliquoted equally into four 1.5 ml Eppendorf tubes and centrifuged for 1 min at 500 rpm, and the supernatant was discarded. The protein incubations described above were added individually to the tubes containing the beads and incubated for 2 h at room temperature with rocking. Beads were subsequently pelleted and washed 3X with 200 µl DPBS+/+10 mM Mg²⁺/1 mM Ca²⁺ (Cambrex Bio Science) containing 1.0% (w/v) BSA and 0.05% (v/v) tween-20. Bead-bound proteins were separated by SDS-PAGE (Criterion XT precast 10% Bis-Tris gel, Bio-Rad, Hercules, CA, USA), transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA), and analyzed for the presence of SPARC via Western blotting with goat anti-hSPARC IgG and bovine anti-goat IgG-HRP conjugate.
SPARC-VCAM-1 affinity assay

hSPARC was isolated as described previously [20] and was spotted (50 ng/100 µl PBS) onto flat-bottom Nunc Maxisorp plates overnight at 4°C. Wells were blocked with PBS/5% BSA and subsequently incubated in triplicate with increasing log concentrations of hVCAM-1 (R&D Systems) for 1 h at room temperature. After incubation, wells were washed 6x with PBS/1% BSA/0.1% Tween-20 and incubated with mouse anti-hVCAM-1/ICD106g (1:50) for 1 h at room temperature, and immune complexes were detected with goat antimouse IgG-HRP (1:1000), developed with TMB (30 min, room temperature). Absorbance was read at 650 nm on a colorimetric plate reader (Emax).

Cell adhesion under flow conditions

Leukocyte adhesion to endothelial monolayers was performed as described previously [21]. Briefly, HUVEC were cultured on coverslips to confluence as described previously [21]. Human PMN and MNC were isolated as described above, diluted to a concentration of 1 x 10⁶ cells/mL in flow buffer (1:1 DPBS/+10 mM Mg₂⁺/1 mM Ca²⁺:DPBS+/10 mM Mg₂⁺/1 mM Ca²⁺ containing 0.1% BSA), and used immediately. Confluent HUVEC were activated with 25 ng/mL iTNF-α (R&D Systems) for 4 h. HUVEC were also incubated for the last 30 min with 5 µg SPARC (Haematologic Technologies) or vehicle control. Human leukocytes were preincubated with 1 µg/mL SPARC or vehicle control for 30 min prior to use in flow experiments. Leukocytes were then perfused across the endothelial monolayer at a flow rate of 0.85 µl/min (estimated wall shear stress of 1.8 dynes/cm²) or 0.52 µl/min (estimated wall shear stress of 1.0 dynes/cm²) for PMN and MNC, respectively, for a total of 10 min. Rolling leukocytes were not considered as adherent after 30 s of stable contact with the endothelial monolayer. Rolling leukocytes were not considered as adherent. Statistical significance was calculated using a standard t-test.

Static transmigration assay

MCEC from SPARC null or wild-type C57Bl/6 mice were plated at 95% confluence on fibronectin-coated, 3 µm Transwell inserts (BD Biosciences, San Jose, CA, USA) and were allowed to grow for 48 h to confluence. MCEC were activated with 120 ng/mL mTNF-α (R&D Systems) 4 h prior to assay. Murine leukocytes were isolated from the spleens of SPARC null or wild-type mice, as described previously, and were used immediately after isolation at a density of 5 x 10⁶ cells/mL. The leukocyte suspension (500 µl) was applied to the upper wells of virgin fibronectin-coated transwells or fibronectin-coated transwells containing confluent monolayers of TNF-α-activated MCEC and was incubated for 1.5 h at 37°C to allow leukocyte migration. Transwells were subsequently removed from the tissue-culture plate, and cells in the lower wells were counted to determine the degree of transendothelial migration.

SPARC-induced cellular changes

HUVEC were cultured in 96-well plates and were incubated with DPBS+/10 mM Mg²⁺/1 mM Ca²⁺ (Cambrex Bio Science; DPBS+) alone or DPBS+ containing mouse anti-hVCAM-1 (10 µg/mL) or mouse anti-hhCAM-1 (10 µg/mL) for 30 min at 37°C. Subsequently, 15 µg/mL SPARC (Haematologic Technologies) or vehicle was added, and incubation proceeded for 4 h at 37°C. Cells were next washed 2x with DPBS+, fixed with 2% paraformaldehyde for 5 min at room temperature, permeabilized with 0.5% Triton X-100 for 5 min at room temperature, and incubated with fluorescein phalloidin (Molecular Probes, Carlsbad, CA, USA; 5 µl 6.6 µM stock in 200 µl DPBS+/1% BSA) for 15 min at room temperature. VectaShield (Vector Laboratories, Burlingame, CA, USA; 20 µl) was added to each well to prevent fluorescence photobleaching, and the cells were photographed on a Nikon Eclipse TE2000-S with a Spot Insight QE charged-coupled device camera, 40x objective.

Time course of SPARC-induced albumin flux

HUVECs were grown to confluence in 1 mL medium on fibronectin-coated, 3 µm Transwell inserts (BD Biosciences) and were allowed to grow for 48 h to confluence. HUVECs were activated with 120 µg/mL hTNF-α for 4 h prior to the assay. FITC-labeled BSA (Molecular Probes; the tracer molecule) was prepared in serum-free media to produce a final protein concentration of 100 µg/mL. hSPARC [20] or vehicle was added to the upper compartment of the transwells containing FITC-BSA-supplemented media for 0, 3, 15, 30, 60, and 240 min. The amount of FITC-BSA in the lower compartment of the chamber was assayed by fluorimetry on a GeminiXS (Molecular Devices) plate reader (ex/em 485/538).

Thioglycollate-induced peritonitis model

Injections (i.p.) of 1 mL 3% thioglycollate broth were given to mice to elicit leukocyte migration into the peritoneal cavity as described previously [22]. Mice were killed and leukocytes harvested by peritoneal lavage with 10 mL cold PBS, 6, 48, or 96 h after injection of thioglycollate. Control mice of each genotype were killed without the administration of thioglycollate to assess the number of resident leukocytes in the peritoneum. Total leukocytes were counted via a S1 Coulter counter (Beckman Coulter, Fullerton, CA, USA). The cellular compositions of lavages were determined by Diff-Quik-stained (WVR, West Chester, PA, USA) cytocentrifuge preparations. A total of 15 mice for each genotype (wild-type or SPARC−/−) was used, and three mice were used per time-point (6, 48, and 96 h) and three mice per genotype for the 0-h control. Statistical significance was calculated using a standard t-test.

RESULTS

To identify peptide motifs with high, constitutive VCAM-1-binding, we have performed several phage screens under physiologic flow conditions (Fig. 1A) [19]. These screens identified

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**Fig. 1.** Phage selection on activated endothelium identifies a peptide family with homology to SPARC. (A) Results of phage display screening on MCEC under physiological flow conditions. The ratio of intracellular- to EG-bound phage on MCEC was saturated by successive rounds of panning, (B) Sequence alignment of putative SPARC-related peptides. Note the consensus sequence in bold. (C) Portions of mouse and hSPARC protein sequence that are homologous to the phage display peptide consensus sequence. Numbers indicate the position of amino acids in the SPARC protein.
VCAM-1-binding peptides with similarity to VLA-4 as expected [19] but also a cluster of peptides (Figs. 1B and 2, A and B) similar in sequence to the calcium-binding EC domain (residues 150–155 of hSPARC NP_003109 and 149-154 of Mus musculus CAJ18514) of the 32-kDa matricellular glycoprotein SPARC (Fig. 1C). Many of the effects of SPARC on cultured cells have been attributed to the EC domain and to the Ca\(^{2+}\)-binding loop [23]. The hypothesis that SPARC may bind to VCAM-1 in vivo was plausible, given the similarity of the sets of events that each protein is known to mediate. Although SPARC has been known to bind to endothelial cells [9] and mediate cytoskeletal rearrangement and vascular permeability [10–14], the receptor-mediated pathways by which these changes occur have not been identified.

To validate the initial phage-display observations, we next determined whether full-length, purified SPARC protein interacted with VCAM-1. Coprecipitation studies, using purified FcVCAM-1, incubated with SPARC protein, demonstrated that FcVCAM-1 interacted with SPARC in a concentration-dependent manner (Fig. 2, C and D). SPARC bound to VCAM-1 in relevant molar quantities (1:3 SPARC:VCAM-1), and an increase in the concentration of SPARC resulted in a corresponding increase in the amount of SPARC protein present in the complex with VCAM-1 (6.9-fold greater compared with 1 μg SPARC; Fig. 2D). The interaction was specific for VCAM-1, as ICAM-1 did not coprecipitate SPARC under identical conditions (Fig. 2, C and D). The interaction of SPARC/VCAM-1 was saturable with a binding affinity of 3.8 μM ± 0.25 (Fig. 2E).

To examine the physiological significance of VCAM-1/SPARC interactions, we investigated the effect of hSPARC on leukocyte adhesion, rolling, attachment, and transmigration in a parallel plate flow chamber, which mimics important features of blood vessel biology [21]. We first evaluated human PMN, as endothelial adhesion of these cells is not mediated through the VLA-4/VCAM-1 axis, thereby allowing us to segregate the effects of SPARC on adhesion versus transmigration. Incubation of HUVECs with SPARC altered the transmigration of PMN significantly but did not alter total interactions or the other parameters of adhesion, rolling, and attachment (Fig. 3A). In contrast to the findings with PMN, total interactions as well as transmigration were reduced significantly by incubation of SPARC with human MNC, which use the VLA-4/VCAM-1 axis for rolling and arrest on endothelial substrates (Fig. 3B).

Fig. 2. SPARC is a counterligand for VCAM-1. (A) ELISA was used to identify phage clones that bound specifically to VCAM-1. Note the high degree of binding for Clones 9 and 20, which are homologous to VLA-4, and Clones 11, 17, and 19, which are homologous to SPARC. (B) Immunofluorescence demonstrating MQKSIPAC phage binding to MCEC through VCAM-1 but not ICAM-1 (40× objective). (C) In in vitro coprecipitation experiments, FcVCAM-1 but not FcICAM-1 binds to SPARC in a concentration-dependent manner. (D) Quantification of bands present in C. (E) Binding of hVCAM-1 to immobilized hSPARC. Increasing log concentrations of hVCAM-1 were incubated in triplicate with hSPARC immobilized onto immunoplates. The presence of hVCAM-1 was assayed by incubation of washed wells with mouse anti-hVCAM-1 IgG and detected with goat antimouse IgG-HRP followed by development with TMB. Absorbance (650 nm) was read on a plate reader.
Previous studies in a carcinoma model had indicated that leukocyte-derived SPARC was required for the development of stroma and EC matrix [24] and that conversely, secretion of SPARC by human melanoma cells modulated the recruitment of neutrophils to the tumor [25]. Thus, we sought to determine the relevance of leukocyte versus endothelial-derived SPARC in modulating leukocyte extravasation in a transmigration assay. The ability of SPARC null or wild-type mouse leukocytes to migrate through TNF-α/H9251-activated endothelial cells was assessed by counting the number of cells that traversed the monolayer. The presence of SPARC on the leukocytes was essential for effective transendothelial migration, as leukocytes derived from SPARC null mice demonstrated significantly reduced transendothelial migration across wild-type or SPARC null endothelium (Fig. 3C), in comparison with leukocytes derived from wild-type animals. Furthermore, leukocytes derived from wild-type mice were equally capable of traversing the endothelium, regardless of the presence or absence of SPARC in the endothelial cells. The ability of the leukocytes to migrate through the fibronectin-coated transwells without an endothelial barrier was the same, regardless of their status with respect to SPARC (Fig. 3D).

To ascertain the functional importance of SPARC on leukocyte recruitment in vivo, we used a well-described mouse model of peritonitis [22]. Injection (i.p.) of thioglycollate induces an inflammatory response, which includes the recruitment of neutrophils, eosinophils, and macrophages at defined time-points. At baseline, the numbers of resident macrophages were similar between the two genotypes. As expected, thioglycollate injection elicited robust accumulation of total leukocytes for wild-type and SPARC−/− mice by 96 h after thioglycollate injection compared with untreated animals. However, at 6 and 48 h postinjection, leukocyte recovery from SPARC−/− animals was only 49% and 64% of that from wild-type mice, respectively (P<0.05, Fig. 4A). To quantitate differences in leukocyte subset recruitment to the peritoneal cavity, we determined cell type by morphological analysis using Diff-Quik-stained cytocentrifuge preparations of peritoneal lavages. In wild-type animals, neutrophil recruitment reached a maximum at 6 h postinjection and dropped sharply by 48 h postinjection, as described previously [22]. In contrast, neutrophil recruitment was inhibited markedly at 6 h, with only 29% as many neutrophils as observed in wild-type animals (P<0.05; Fig. 4B). Eosinophil recruitment was affected similarly in SPARC−/− mice, with only 38% as many eosinophils recovered at 48 h, which is the peak time-point for eosinophil recruitment (Fig. 4C). There was also a trend toward decreased macrophage recruitment at the 48- and 96-h time-points (Fig. 4D). These data are consistent with the hypothesis that SPARC contributes to efficient leukocyte transmigration.
Previous studies of endothelial cell exposure to SPARC demonstrated SPARC-induced actin cytoskeletal rearrangement and the appearance of intercellular gaps [10]. SPARC has also been shown to mediate actin fiber reorganization and the appearance of intercellular gaps that correspond to increases in the endothelial barrier permeability, although the receptor(s) on endothelial cells mediating these effects have remained elusive [10, 14]. Therefore, we determined whether interaction of SPARC with VCAM-1 mediated these effects. HUVEC monolayers were pretreated with vehicle (PBS), anti-VCAM-1, or anti-ICAM-1 antibodies, followed by incubation with media alone or media containing SPARC. The endothelial monolayers were stained with fluorescein-phalloidin to reveal the actin filaments (Fig. 5, A–D). All control HUVECs (no added SPARC) exhibited continuous transcytoplasmic actin (arrow, Fig. 5A) and tight cellular apposition with few intercellular gaps. In contrast, SPARC-treated HUVECs demonstrated increased peripheral actin bands and the presence of intercellular gaps (arrowheads, Fig. 5, B and D). The preincubation of HUVEC with anti-VCAM-1 protected endothelial monolayers against SPARC and resulted in a significant decrease in the appearance of intercellular gaps (P<0.0001) and the reorganization of actin fibers (Fig. 5, C and E). This effect was VCAM-1-specific, as preincubation with the identical concentration of anti-ICAM-1 antibody had little or no effect on SPARC-induced cellular changes (Fig. 5, D and E). We thus demonstrate that blocking the VCAM-1 receptor abrogates SPARC-induced cell rounding, cytoskeletal reorganization, and the appearance of intercellular gaps. Given that intercellular gaps have been shown to correlate with a compromised endothelial barrier function, we quantified and explored the time course of a SPARC-induced junctional opening. The effect of SPARC on endothelial barrier function was measured with intact TNF-α-activated HUVEC monolayers and FITC-labeled BSA as a tracer molecule. As shown in Figure 5F, SPARC compromised endothelial barrier function within 3 min of its addition to the cells.

DISCUSSION

In the present experiments, we have used an unbiased approach to search for novel binding partners for the endothelial adhesion protein VCAM-1, which has important roles in atherogenesis, tumor growth, and other inflammatory pathways [5, 26]. The data we present demonstrate for the first time the interaction of leukocyte-derived SPARC with endothelial cell VCAM-1. Through phage-display screening, we identified peptides that bound to VCAM-1 and resembled sequences of SPARC. Further experiments with purified SPARC and VCAM-1 demonstrated that SPARC indeed binds specifically to VCAM-1, with an affinity of 3.8 μM, thereby confirming an
interaction of these two previously unrecognized binding partners. Our findings are supported by earlier published reports that SPARC binds to a receptor on endothelial cells with a molecular weight similar to that of VCAM-1 and modulates events that have also been attributed to VCAM-1, such as cytoskeletal rearrangement and vascular permeability [9, 12].

The affinity of the interaction between SPARC and VCAM-1 is comparable with that of several integrins for their ligands and is consistent with an intermediate state of cellular adhesion. An intermediate state of adhesion has been suggested to favor cell motility, as strong adhesion prevents cells from releasing, whereas weak adhesion provides insufficient contractile force necessary for cellular movement [27].

SPARC has also been shown to mediate actin fiber reorganization and the appearance of intercellular gaps that correspond to increases in the endothelial barrier permeability [10, 14]. In this work, we demonstrate that the interaction of VCAM-1 with SPARC is responsible for these effects and that
blocking the VCAM-1 receptor with an anti-VCAM-1 antibody abrogates SPARC-induced cell rounding, cytoskeletal rearrangement, and the appearance of intercellular gaps. The capacity of the VCAM-1 antibody to block the SPARC-induced changes is likely a result of steric hindrance but is specific to VCAM-1, as an anti-ICAM-1 antibody was unable to block these physical changes. We demonstrate further that incubation with SPARC compromises endothelial barrier permeability over a time course relevant to leukocyte transmigration. SPARC significantly decreased the transmigration of leukocytes across endothelial monolayers under flow conditions. In static transmigration assays, we determined that leukocyte-derived SPARC is essential for diapedesis. Furthermore, we demonstrate in vivo that SPARC is necessary for leukocyte recruitment to the peritoneal cavity in a model of peritonitis. Taken together, these experiments show that the interaction of leukocyte-derived SPARC with VCAM-1 contributes to efficient leukocyte transmigration.

We show that SPARC on leukocytes interacts with VCAM-1 and is associated with cytoskeletal rearrangement and intercellular gaps. The appearance of gaps is coincident with an increased permeability of the endothelial barrier [10], data indicating that SPARC through VCAM-1 plays a role in modulating vascular permeability to allow leukocytes to migrate across the endothelium. That transendothelial migration occurs through junctions [28, 29] supports a role for the SPARC/VCAM-1-induced junctional opening demonstrated in this study. This function in all probability is not mediated by VLA-4, as VLA-4 has been shown to mediate adhesion but not transendothelial migration [8]. Furthermore, SPARC does not significantly affect adhesion of PMN, which primarily use selectins and mucosal addressin/ICAM-1 for adhesion [30]; however, transendothelial migration of PMN is affected by SPARC. In contrast, MNC, which use VLA-4/VCAM-1 for adhesion, are deficient in adhesion and endothelial transmigration, a result indicating that excess SPARC interferes with the VLA-4/VCAM-1 interaction and thereby limits adhesion of leukocytes to the endothelial monolayer. The interference of SPARC with the VLA-4/VCAM-1 interaction indicates that SPARC is under tight regulation under basal conditions. Indeed, SPARC is expressed minimally under homeostatic conditions but is up-regulated substantially upon injury and pathological responses to injury and tumor growth [23]. A consequence of SPARC dysregulation is a decrease in PMN recruitment, which provides a mechanism for tumors to evade host response through the inhibition of antitumor PMN activity [25]. In addition, lack of SPARC expression results in decreased invasion of the tumor by mature macrophages [31].

In this report, we have identified a novel interaction of VCAM-1 with SPARC, which is responsible for cell rounding, actin cytoskeletal rearrangement, the appearance of intercellular gaps, and an increase in endothelial barrier permeability in a time-frame relevant to transmigration. This interaction contributes to efficient leukocyte endothelial transmigration. The elucidation of the interaction of SPARC/VCAM-1 is a significant finding, as leukocyte infiltration is critical for the progression of atherosclerosis and tumorigenesis [4, 32], diseases associated with high levels of mortality. This interaction has widespread implications for the fields of leukocyte transmigration, atherosclerosis, and cancer and presents a possible point of therapeutic intervention for these diseases.

REFERENCES


