Sphingosine 1-Phosphate-induced Endothelial Cell Migration Requires the Expression of EDG-1 and EDG-3 Receptors and Rho-dependent Activation of $\alpha_{v}\beta_{3}$- and $\beta_{1}$-containing Integrins*

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Sphingosine 1-phosphate (SPP), a platelet-derived bioactive lysophospholipid, is a regulator of angiogenesis. However, molecular mechanisms involved in SPP-induced angiogenic responses are not fully defined. Here we report the molecular mechanisms involved in SPP-induced human umbilical vein endothelial cell (HUVEC) adhesion and migration. SPP-induced HUVEC migration is potently inhibited by antisense phosphothioate oligonucleotides against EDG-1 as well as EDG-3 receptors. In addition, C3 exotoxin blocked SPP-induced cell attachment, spreading and migration on fibronectin-, vitronectin- and Matrigel-coated surfaces, suggesting that endothelial differentiation gene receptor signaling via the Rho pathway is critical for SPP-induced cell migration. Indeed, SPP induced Rho activation in an adherence-independent manner, whereas Rac activation was dispensable for cell attachment and focal contact formation. Interestingly, both EDG-1 and -3 receptors were required for Rho activation. Since integrins are critical for cell adhesion, migration, and angiogenesis, we examined the effects of blocking antibodies against $\alpha_{v}\beta_{3}$, $\beta_{3}$, or $\beta_{1}$ integrins. SPP induced Rho-dependent integrin clustering into focal contact sites, which was essential for cell adhesion, spreading and migration. Blockage of $\alpha_{v}\beta_{3}$ or $\beta_{1}$-containing integrins inhibited SPP-induced HUVEC migration. Together our results suggest that endothelial differentiation gene receptor-mediated Rho signaling is required for the activation of integrin $\alpha_{v}\beta_{3}$ as well as $\beta_{1}$-containing integrins, leading to the formation of initial focal contacts and endothelial cell migration.

Activation of the sphingomyelinase enzyme followed by sequential catalysis by ceramidase and sphingosine kinase results in the formation of SPP (1). Although the site of synthesis and mechanisms involved in secretion of SPP are not well understood, it is clear that one mechanism by which SPP acts is via the interaction with plasma membrane-localized G-protein-coupled receptors (GPCR) of the EDG family (2, 3). To date, EDG-1, -3, -5, -6, and -8 were shown to bind to SPP and transduce various intracellular signals (4–9). Signal transduction mechanisms of EDG-1, -3, and -5 have been defined. These GPCRs are stimulated by nanomolar concentrations of SPP and Couple to different G-proteins, which may form the basis for their differential signal transduction properties (5, 6, 10). For example, EDG-1 couples to the G protein, whereas EDG-3 and -5 couple to $G_{q}$, $G_{13}$, and G proteins (10). Downstream of the heterotrimeric G-proteins, small GTPases of the Rho family, namely Rho and Rac, are activated by SPP (11, 12). Rho and Rac regulate cytoskeletal changes such as stress fiber assembly and cortical actin formation, respectively (13). Based on changes in actin dynamics and using antisense oligonucleotides to block the expression of EDG-1 and -3, we proposed that EDG-1 is required for cortical actin assembly (a Rac-regulated event) and EDG-3 for stress fiber formation (a Rho-regulated event) (12). However, complex cross-regulatory mechanisms appear to be involved since dominant negative Rac also blocks stress fiber assembly in endothelial cells (12, 13). Direct measurements of Rho and Rac activity induced by SPP in endothelial cells has not been reported.

SPP acts in a variety of cell systems to regulate cell proliferation, migration, differentiation, and death (1–3, 14, 15). Although controversy exists regarding its mode of action, i.e. whether SPP is a second messenger that acts intracellularly or a first messenger that acts extracellularly, many of the aforementioned actions are likely to be due to activation of EDG family of receptors (1–3, 14, 15). Furthermore, physiological functions of SPP are poorly understood.

EDG-1, the prototypical SPP receptor, was originally isolated from human umbilical vein endothelial cells (HUVECs) as an inducible gene (3, 16). SPP treatment of HUVECs results in activation of EDG-1 and -3 receptors, stimulation of the G-dependent cell survival pathway, Rac- and Rho-dependent adherens junction assembly, and cytoskeletal rearrangement, which ultimately results in the morphogenesis of HUVECs into capillary-like networks (12). Indeed, SPP synergized with polypeptide angiogenic factors such as fibroblast growth factor-2 and vascular endothelial cell growth factor to induce mature neovessels in vivo (12). Recent data on the deletion of the Edg-1 gene in the mouse indicate that SPP/Edg-1 interaction is necessary for embryonic vascular maturation (17). Interestingly, vasculogenesis and angiogenesis occurred in the...
Edg-1−/− embryos, suggesting that Edg-1 is dispensable for these processes during embryonic development. Interestingly, genetic studies in zebrafish indicate that an EDG-5-like receptor (termed as Miles Apart) controls myocardial progenitor cell migration and heart development (18). These data suggest that a physiological function of SPP is to regulate the development, growth, and maintenance of the cardiovascular system.

Recently, several studies reported that SPP is a potent migration inducer of vascular endothelial cells (19–24, 47). Previously, however, SPP has been shown to both stimulate and inhibit chemotactic responses in a variety of cell types. CHO cells expressing EDG-1 or EDG-3 showed better migration toward SPP, whereas Edg-5-expressing cells did not (24). On the other hand, SPP inhibited chemoinvasiveness of MDA-MB-231, a human breast cancer cell line (25). Moreover, chemotactic motility of mouse melanoma has been shown to be inhibited by SPP in a pertussis toxin-sensitive G protein-independent way (26). Molecular mechanisms of SPP-induced endothelial cell migration have not been defined.

Here we investigated the mechanisms involved in SPP-induced migration and morphogenesis of HUVECs. Data in this report show that SPP is effective in stimulating the migration and lumen formation of HUVECs. This involves rapid assembly of the initial focal contacts through αβ2 and β1 family of integrins. Furthermore, we demonstrate that activation of Rho by SPP through EDG-1 and EDG-3 in HUVECs is required for this process.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**HUVECs (Clonetics) were cultured in M199 medium (Cellgro, Inc.) supplemented with 10% fetal bovine serum (HyClone) and heparin-stabilized endothelial cell growth factor, as previously described (16). CHO cells (pCDN-EDG-1, EDG-3) were maintained in F-12 medium with 10% fetal bovine serum and 1 mg/ml G418. Transfection of antisense oligonucleotides to block the expression of EDG-1 and EDG-3 was performed using NovaFEC-TOR™ reagent (VennNova, LLC, Pompano Beach, FL). HUVECs were plated the day before transfection in complete growth medium. 18-mer phosphorothioate oligonucleotides (PTO; antisense EDG-1, 5'-GAC GCT GGT GGC CCC ACC AGC-3'; sense EDG-1, 5'-GGG CCC ACC AGC GTC-3'; antisense EDG-3, 5'-GGG GAG GGC AGT TGC CAT-3'; sense EDG-3, 5'-ATG GCA ACT GCC CTC CCG-3') were mixed with NovaFECTOR™ in 200 μl of OPTI-MEM® I and left for 5 min to allow DNA-lipid complex formation. Cells were rinsed three times with pre-warmed OPTI-MEM®, and the DNA-lipid complex was layered over cells at the final concentration of 400 μl. After 4 h of transfection, medium was removed and replaced with complete growth medium.

**Northern Blot Analysis—**Total RNA was isolated from HUVECs using the RNA-STAT 60 (Tel-test) following the manufacturer's instructions. Fifteen micrograms of total RNA were resolved on 1% agarose-formaldehyde gels, and the integrity of the RNA was monitored by ethidium bromide staining. RNA was capillary-transferred onto Zeta-Probe membrane (Bio-Rad), UV-cross-linked by Stratalinker, then hybridized with radiolabeled cDNA probes. The human EDG-1, EDG-3, and glyceraldehyde-3-phosphate dehydrogenase cDNAs were radioactively labeled using [α-32P]dCTP (Amersham Pharmacia Biotech) by a random primer labeling system (Roche Molecular Biochemicals). The hybridized membranes were washed as described (16) and visualized by autoradiography. The bands were quantified using Image Quant (Molecular Dynamics).

**Microinjection—**HUVECs were grown on fibronectin-coated glass bottom dishes and microinjected using an Eppendorf Micromanipulator 5171 and Transjector 5246. Approximately 500 cells were microinjected cytoplasmically with 0.1 μg/ml C3 exoenzyme (Calbiochem) or 0.8 μg/ml dominant-negative N17Rac protein (12) with Femtotips (Eppendorf) at 100 hepatopacaps/0.2 s. Injected cells were marked by coinjection of 5 mg/ml Texas Red®-labeled dextran (Molecular Probe) (12).

**Migration Assay—**Cell migration assays were performed using 24-well chemotaxis chambers (Nalgene). Polycarbonate filters with a pore size of 8 μm (Nalgene) were coated with 5 μg/ml fibronectin, 200 μg/ml Matrigel (Becton Dickinson), or 5 μg/ml bovine vitronectin (Life Technologies) at 4 °C overnight in phosphate-buffered saline (PBS) and dried under sterile air. SPP and/or inhibitors were diluted to appropriate concentrations in M199 supplemented with 0.5% fatty acid free bovine serum albumin (Sigma), and 600 μl of the final dilution was placed in the lower chamber of a modified Boyden chamber. HUVECs were washed with PBS and trypsinized for the minimum time required to achieve cell detaching. Approximately 5 × 104 cells suspended in 100 μl of M199 with 0.5% bovine serum albumin were plated in the upper chamber of the Boyden chamber. The cells were allowed to migrate for 3 h at 37 °C in a humidified chamber with 5% CO2. After the incubation period, the filter was removed, and the nonmigrated cells on the upper side of the filter were removed with a cotton swab. The filters were fixed with 4% formaldehyde and stained with hematoxylin. Migration was quantified by counting cells in three random high power fields (100×) for each filter. Alternatively, filters were stained in 0.1% crystal violet and eluted with 10% acetic acid in 96-well plates. Quantification was done by determining absorbance (A) at 575 nm by a Spectramax 340 (Molecular Devices) plate reader.

**Attachment Assay—**Antibodies against integrins were purchased from Chemicon. Ninety-six well plates were coated with FN (5 μg/ml), VN (5 μg/ml, Life Technologies), or MG (200 μg/ml, Beckton Dickinson) at 4 °C overnight. Plates were blocked with 10 mg/ml heat-denatured fatty acid free bovine serum albumin for 30 min. HUVECs (5 × 104 cells/ml in M199 with 0.1% fatty acid free bovine serum albumin) were incubated with function-inhibiting anti-integrin antibodies (β1, β3, LAMA4) or other antibodies for 10 min before plates. Then 100 μl of the cell suspension/well was added onto plates and incubated for 30 min at 37 °C under 5% CO2 with the lid off. After incubation, nonspecifically attached and unattached cells were removed by rinsing with PBS. Attached cells were fixed in 5% glutaraldehyde for 20 min and stained with 0.1% crystal violet. Stains were dissolved in 10% acetic acid, and plates were read at A570 nm by a Spectramax 340 plate reader.

**Immunocytochemistry—**Cells were rinsed with ice-cold PBS and fixed in 3.7% formaldehyde for 15 min at room temperature followed by permeabilization with 0.1% Triton X-100. Anti-paxillin antibody (1:100, Transduction Laboratories) in PBS was incubated for 90 min at room temperature. The primary antibody staining was visualized with FITC-conjugated goat anti-mouse (1:200, Jackson Laboratories). Actin cytoskeleton was stained with either FITC- or tetramethylrhodamine B isothiocyanate-phalloidin (0.05 μg/ml, Sigma) for 30 min at room temperature. Images were observed with a Zeiss Axiosvert fluorescence microscope and captured by a SPOT™ digital camera (Diagnostic Instruments, Inc).

**Affinity Precipitation of Rho-GTP/Rac-GTP and Immunoblotting—**HUVECs were washed with ice-cold PBS and lysed in radioimmune precipitation buffer (50 mM Tris, pH 7.5, 1, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 1 μg protease inhibitor mixture), and cell lysates were cleared by centrifugation at 20,000 × g at 4 °C for 10 min. Equal amounts of lysates were incubated with GST-C21 (Rho binding domain of Rhotekin quanine nucleotide exchange factor, kindly provided by Dr. Shuh Narumiya, Kyoto University; JA2, an Fab fragment of an anti-RhoA binding domain of p21-activated kinase, kindly provided by Dr. Martin Schwartz, The Scripps Research Institute, La Jolla, CA) beads at 4 °C for 1 h. The beads were washed three times with wash buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 1 μg protease inhibitor mixture). Bound proteins were resolved on 12% SDS-polyacrylamide gel electrophoresis and detected by Western blot analysis using a monoclonal antibody against Rho (Santa Cruz Biotechnology) or Rac1 (Transduction Laboratories). Equal loading was confirmed by blotting against Rho, Rac, or β-actin (Sigma).

**Endothelial Cell Morphogenesis Assay in Three-dimensional Fibrin Gel—**This assay was performed as described in Bayless et al. (27) with minor modifications. HUVECs were rinsed with PBS, trypsinized, and resuspended in Dulbecco’s minimum essential medium (Cellgro) with 5 μg/ml soybean trypsin inhibitor (Sigma). Cells were rinsed twice with Dulbecco’s minimum essential medium and resuspended at a density of 1 × 105/ml. Fibrinogen was added as a final concentration of 10 mg/ml and polymerization was achieved by adding lumi/ml thrombin. Fibrin gel was covered with a volume of Dulbecco’s minimum essential medium containing 20% charcoal-stripped serum, heparin-stabilized endothelial cell growth factor, and different concentrations of SPP along with integrin antagonists. After an 18-h incubation at 37 °C with 5% CO2, medium was removed, and gels were fixed with 3% glutaraldehyde in PBS. Pictures were taken from several random fields of each well. Gels were embedded into paraffin block and sectioned for hematoxylin staining.
EDG-1 and EDG-3 Are Required for SPP-stimulated Migration of HUVECs—SPP was previously shown to induce endothelial cell migration; however, mechanisms involved are not clearly defined (19–24). We and others have shown that HUVECs express EDG-1 and -3 subtypes of GPCRs for SPP (12, 19–24). To define the role of SPP receptors in endothelial cell migration, we utilized the antisense PTOs, which block the expression of EDG-1 and -3 GPCRs (12). HUVECs were transfected with sense or antisense PTOs against EDG-1 or -3 using the lipiod-mediated method. Expression of EDG-1 and -3 receptors was assayed by Northern analysis. As shown in Fig. 1A, treatment with EDG-1 antisense PTO specifically reduced the steady-state mRNA levels for EDG-1 and not EDG-3. Likewise, antisense EDG-3 PTO blocked expression of EDG-3 mRNA in a specific manner. Quantitative analysis indicated that antisense PTO for EDG-1 (a1) showed >90% suppression of EDG-1 mRNA levels. Antisense PTO for EDG-3 (a3) had almost 60% reduction of EDG-3 mRNA levels (Fig. 1A), whereas sense counterparts for a1 and a3 had no effect. These data indicate that the antisense EDG-1 and -3 PTOs are efficacious and selective in inhibiting the expression of respective receptors.

When treated with SPP, HUVECs migrated on different extracellular matrices (ECMs) such as FN, VN, and MG. SPP induced ~6-fold (FN), ~22 fold (VN), ~25 fold (MG) stimulation of migration above the base-line rate in all three matrices (Fig. 1B). However, the basal rate of cell migration was higher in FN-coated dishes, even though SPP stimulated further chemotaxis. SPP-induced HUVEC migration was dramatically inhibited by the antisense EDG-1 and -3 PTOs, suggesting that each of these GPCRs for SPP is required for chemotaxis.

SPP induced a dose-dependent increase of HUVEC migration over the range of 10 nM–0.5 μM (data not shown). Cell migration responses were inhibited by pretreatment with C3 exotoxin (4 days, 5 μg/ml), which inhibits the small GTPase Rho (Fig. 2A). These data suggest that EDG receptor signaling via the Rho pathway is essential for SPP-induced endothelial cell migration.

The Rho GTPase is activated by extracellular mediators and regulates the formation of actin stress fibers and focal adhesion sites (28, 13). When HUVECs were plated on FN, VN, or MG and treated with SPP for 15 min, both focal adhesion sites and stress fibers were induced (Fig. 2B and data not shown). Thus, cell matrix adhesion and spreading were induced by SPP. FN supported basal adhesion of HUVECs in the absence of stimulation and further enhanced adhesion, stress fiber formation, and focal contact site assembly after SPP addition (data not shown). Although VN did not support basal adhesion in the absence of the ligand, SPP strongly induced adhesion, spreading, and focal contact assembly on cells plated on VN (Fig. 2B). Although these events occurred on Matrigel, a complex extracellular matrix enriched in laminin (29), only small focal contact sites and stress fibers were induced by SPP (data not shown). Treatment of cells with C3 exotoxin strongly inhibited SPP-induced focal contact site assembly and stress fiber formation (Fig. 2B). Similarly, microinjection of C3 exotoxin also blocked focal contact site assembly (Fig. 2C). Interestingly, microinjection of dominant negative Rac (N17Rac) protein did not inhibit SPP-induced focal contact formation and cell spreading (Fig. 2C). These data suggest that SPP/EDG receptor signaling induces a Rho signal that is essential for initial focal contact assembly and ultimately for cell migration.

Regulation of Rho and Rac by SPP in HUVECs—To directly measure the effect of SPP on Rho and Rac activation, we utilized the GST pull-down assays for Rho and Rac GTPases (28, 30, 31). As shown in Fig. 3A, strong activation of Rho by SPP was observed in HUVECs plated on both VN and FN. Rac was also induced, although the magnitude of stimulation was less than that of Rho. Interestingly, basal Rac activity was enhanced in FN, which supports better HUVEC adhesion under basal conditions. SPP effect was more pronounced in HUVECs plated on VN than FN. These data indicate that SPP directly stimulates Rho and Rac GTPases in HUVECs.

Since SPP induced focal contact formation in nonadherent HUVECs in a C3-inhibitable manner, we tested the effect of SPP on Rho and Rac activation in nonadherent conditions. HUVECs were detached from the culture dish and held in suspension with 100 mM SPP for 0–60 min at 37 °C. At each time point, cells were harvested and analyzed for the level of active Rho and Rac. As shown in Fig. 3B, SPP strongly stimu-
lated Rho activity of HUVECs in suspension within 10–20 min (upper panel). In contrast, SPP did not significantly affect Rac activity (Fig. 3B, lower panel) of nonadherent HUVECs. These data indicate that SPP induces Rho activity in an adherence-independent manner, which is required for initial focal contact assembly.

Regulation of Rho and Rac by EDG-1 and EDG-3—We next addressed the involvement of EDG-1 and -3 receptors in the activation of Rho and Rac by SPP. Cells were pretreated with the antisense PTO of EDG-1 or EDG-3 and then stimulated with 100 nM SPP. As shown in Fig. 4, antisense PTO against EDG-3 strongly inhibited the SPP-induced Rho activation.
However, antisense PTO against EDG-1 also inhibited Rho activation, suggesting that both receptors contribute to Rho activation. Similarly, antisense EDG-1 and EDG-3 blocked Rac. These data suggest that both EDG-3 and -1 receptors contribute to SPP-induced Rho and Rac activation.

To confirm the antisense PTO blockage data, we utilized the CHO cell system, which exhibited a very low endogenous SPP receptor activity and expression (23, 32). CHO cells stably transfected with EDG-1 or -3 receptors or vector-transfected controls were stimulated with SPP, and Rho and Rac activity was directly measured. As shown in Fig. 4D, both Rho and Rac were activated in both EDG-1 and -3-transfected CHO cells. These data confirm the antisense PTO inhibition experiments in HUVECs and suggest that both EDG-1 and -3 are capable of activating Rho as well as Rac-dependent signaling pathways.

SPP-induced HUVEC Migration Is Inhibited by Integrin Inhibitors—Focal contact sites are formed by activation and clustering of various integrins and associated molecules (33). It is known that β3- and β1-containing integrins, namely, αvβ3, αvβ1, and α5β1, are critical for endothelial cell adhesion, migration, and morphogenesis (34–36). To assess the role of different integrins in SPP-induced HUVEC migration, we utilized blocking antibodies for β1 and β3 αvβ3 as well as echistatin, a disintegrin antagonist of αvβ3 integrin (37). As shown in Fig. 5A, HUVEC adhesion was stimulated by SPP potently in VN and MG and less potently in FN. These data are consistent with the finding that FN supports basal adhesion to HUVECs more than VN and MG. Antibodies to β3 integrins strongly inhibited basal and SPP-induced adhesion in FN and MG. This is consistent with the knowledge that HUVECs adhere to FN via the αvβ3 integrin and adhere to MG via the laminin receptor α5β1 (35, 36). Blockage of β3 integrins with anti-β3 antibody, LM609, or echistatin did not influence basal or SPP-induced cell adhesion on FN and MG. In contrast, HUVEC adhesion to VN is strongly inhibited by anti-β3 antibody, LM609, and echistatin, suggesting that the αvβ3 integrin is involved. These data suggest that SPP activates the β3- as well as β3-type of integrins, particularly α5β1, to induce cell adhesion. SPP-induced cell adhesion and not basal adhesion was strongly inhibited by the C9 exotoxin, suggesting the involvement of Rho activation.

We next conducted HUVEC migration assays on the mixed matrix of FN, VN, and MG along with the integrin-blocking reagents. As depicted in Fig. 5B, the addition of anti-integrin β1, β3, and α5β1 antibodies to the migration medium suppressed the SPP-induced HUVEC migration on the mixture of FN, VN, and MG. Echistatin, a disintegrin known to inhibit the binding of αvβ3 to VN and rather weakly α5β1 to FN (37),
abolished the SPP-induced HUVEC migration on the mixed matrix. These data indicate that SPP-induced activation of \( \alpha_v\beta_3 \) and \( \beta_1 \)-type integrins, especially \( \alpha_v\beta_3 \), are critical for the stimulation of HUVEC migration.

SPP-induced Endothelial Cell Morphogenesis in Three-dimensional Fibrin Matrix Requires \( \alpha_v\beta_3 \) Integrins, as Well as the Activity of Rho—Bayless et al. (27) demonstrate that HUVECs undergo morphogenetic differentiation into lumenal structures in a three-dimensional fibrin matrix in the presence of phorbol 12-myristate 13-acetate. Recently it has been shown that SPP can induce endothelial morphogenesis on MG (12) and collagen (21). We next determined the requirement for integrins in SPP-induced morphogenesis of HUVECs in the fibrin matrix. HUVECs were set to differentiate into tubular structures in the absence (Fig. 6A) or presence (Fig. 6, B–H) of 500 nM SPP along with a combination of anti-integrin antibodies or C3 (5 \( \mu \)g/ml, 4 days pretreatment). Cells given SPP alone (Fig. 6B) or SPP with control IgG (Fig. 6C) formed luminal structures. On the other hand, function-blocking antibodies against \( \beta_1 \) (Fig. 6F), \( \beta_3 \) (Fig. 6D), \( \alpha_v\beta_3 \) (Fig. 6 E) as well as C3 (Fig. 6G) and echistatin (Fig. 6H) suppressed SPP-induced morphogenesis. These data suggest that SPP-induced Rho-dependent focal contact assembly, integrin activation, and migration are required for HUVEC morphogenesis into luminal structures, an essential step in angiogenesis.

DISCUSSION

Angiogenesis or new blood vessel formation is a critical process required for a variety of physiological processes such as wound healing, embryonic development, and maintenance of the reproductive system (38). Coordinated regulation of endothelial cell migration, proliferation, and assembly is essential in angiogenesis. For coordinated migration, both soluble factors and ECM-derived cues are thought to be important (39). Growth factors such as platelet-derived growth factor, vascular endothelial cell growth factor, and fibroblast growth factor as well as ECM molecules including FN, collagen, fibrin, VN, and laminin are important in regulating different aspects of angiogenesis (40). ECM molecules interact with integrins, which are heterodimeric receptors required for cell adhesion, migration, and morphogenesis. Endothelial integrins have been attractive targets for the inhibition of angiogenesis since \( \alpha_v\beta_3 \) (41) as well as \( \beta_1 \)-containing integrins (42) are implicated in angiogenic responses. \( \beta_1 \)-Containing integrins are important in endothelial cell tube formation in three-dimensional collagen matrix (35). In addition, \( \alpha_v\beta_3 \) has been shown to be up-regulated in angiogenic blood vessels (41). Moreover, \( \alpha_v\beta_3 \) functions in a synergistic manner with receptor tyrosine kinases such as platelet-derived growth factor receptor and vascular endothelial cell growth factor receptor for cell migration and prolifer-
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These data support the notion that SPP-induced signaling pathways induce integrin activation. Indeed, SPP induced focal contact site assembly on all three ECM surfaces in a Rho-dependent manner. Interestingly, Rac activity was not required for initial focal contact assembly and cell spreading.

The Rho family of GTPases are critical regulators of cell motility, cytoskeletal dynamics, cell-cell adhesion, and cell-matrix adhesion (46). SPP induced changes in focal adhesion assembly, and actin changes in fibroblasts and endothelial cells require the activity of the Rho GTPases. Specifically, we showed that SPP-induced actin stress fibers were blocked by the C3 exotoxin, and both cortical actin formation as well as stress fibers were blocked by the dominant negative Rac protein (12). Although these studies indirectly implicate the role of Rho and Rac in SPP-induced events, direct measurement of Rho and Rac activity has not been reported. In this study we show that SPP induces both Rho and Rac GTPases in HUVECs in a rapid manner. SPP induced Rho in nonadherent HUVECs, whereas Rac activation required cell attachment on ECM. This is consistent with the finding that SPP induced cell attachment and spreading to ECM surfaces by inducing focal contact site assembly that was inhibited by the C3 exotoxin. These data are consistent with the work on Rho regulation by serum, lysophosphatidic acid, and SPP in murine fibroblasts, in which the authors showed that serum factors, cell adhesion, and the cytoskeletal structures all contribute to Rho activity (28).

Interestingly, inhibition of EDG-1 and -3 expression with antisense PTOs resulted in the attenuation of SPP-induced Rho and Rac activity. These data are consistent with the fact that both EDG-1 and -3 antisense PTOs inhibited SPP-induced migration and suggest that cooperative signaling of EDG-1 and -3 receptors occurs in HUVECs. Surprisingly, each of the receptors was capable of activating Rho and Rac in transfected CHO cells. Indeed SPP induced migration of CHO cells expressing EDG-1 or EDG-3 (data not shown). This apparent discrepancy between HUVECs and CHO transfectants may be due to cell type-specific differences in signaling pathways. The issue of cooperative signaling between EDG-1 and -3 needs to be further explored. A second issue that needs to be further dissected is the induction of Rac and Rho by both EDG-1 and -3. Previous studies that monitored actin changes suggest that EDG-1 preferentially couples to the Rac pathway, whereas EDG-3 couples to the Rho pathway (12). It is important to stress that such conclusions were based on indirect measurements of Rac and Rho activity. However, direct measurement of Rac and Rho activity by biochemical assays in this report indicates that both receptors are capable of activating both Rac and Rho in both HUVECs (by antisense inhibition studies) and in CHO cell transfectants. Further studies should define the intermediate steps between EDG-1/-3 and Rac/Rho GTPases in different cell types.

SPP-induced focal contact assembly is required for cell spreading and migration. Our data show that β3 integrins are used to attach and spread on FN and MG, whereas αβ3 was responsible on the VN ECM surface. Activation of both types of integrins are important in HUVEC migration induced by SPP since blocking antibodies to β3 and αβ3 as well as echistatin attenuated migration on mixed ECM surface. Furthermore, antagonists to αβ3 and β3 integrins inhibited SPP-induced endothelial cell morphogenesis in a three-dimensional fibrin matrix. These data strongly suggest that SPP/EDG receptors cooperate with αβ3 and β3 integrins to induce HUVEC migration and morphogenesis. Previous work indicates that SPP induces VE-cadherin assembly into adherens junctions, which was necessary for HUVEC morphogenesis on Matrigel (12). Relationships between SPP-induced cell-cell junctions and in-

Results from our lab (12) as well as others (19) implicate that SPP is a regulator of angiogenesis. SPP released during platelet activation accounts for the majority of the angiogenic activity in serum (48). Of interest, numerous groups report that SPP induces the migration of endothelial cells in vitro (19–24). However, molecular mechanisms involved are not well defined. Our data indicate that antisense PTO-mediated suppression of EDG-1 and -3, both, attenuated the SPP-induced migration of HUVECs, suggesting that both receptors are involved. SPP was proposed to be a dual messenger, i.e., an extracellular messenger that interacts with cell surface receptors and an intracellular second messenger that regulates intracellular events (1–3, 14, 15). Data in this study suggest that HUVEC migration is mediated by EDG-1 and -3 GPCRs.

HUVEC migration on a variety of ECM surfaces were induced by SPP, suggesting that SPP regulates the activity of several types of integrins. HUVECs express a variety of integrins including αβ5 (VN receptor), αβ1 (laminin receptor), and ανβ3 (FN receptor) (34–36, 41, 42). αβ3 and β3 integrins have been implicated as critical factors of angiogenesis (41, 42). However, gene deletion studies indicate that αβ3 is dispensable for embryonic angiogenesis (45).

Fig. 6. Inhibition of SPP-induced HUVEC morphogenesis by antagonists of αβ3 or β3. HUVECs were pre-incubated with 30 µg/ml IgG1 (C), 30 µg/ml β3 antibody (D), 25 µg/ml LM609 (E), 30 µg/ml anti-β3 antibody (F), 5 µg/ml C3 (G), or 100 nM echistatin (H). Cells were mixed with unpolymerized fibrin solution, allowed to gel, and treated with 500 nM SPP (B–G) or not (A) for 18 h. Cells were fixed, and cellular morphology was photographed. Arrows denote a tubular structure (white arrows) or regressed structure (black arrows). A sectioned structure of a vacuole stained by hematoxylin is shown in A and B (insets). Bar, 10 µm.
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tegrin-based focal contact site assembly need to be investigated in the future.

In summary, data in this report demonstrate that αβ3 and β1 integrins are required for SPP-induced migration of HUVECs, and SPP-induced activation of Rho through EDG-1 and EDG-3 is required for this process. SPP signaling via the EDG-1 receptor is required for embryonic vascular system maturation in vivo (17), and SPP modulates angiogenesis in the adult (12). SPP-induced activation of integrins may be critical in such events.

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