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Award Number: DAMD17-99-1-9188

TITLE: Regulation of Cell Survival in Human Breast Cancer Cells by Sphingosine Kinase

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REPORT DATE: January 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2001	Annual Summary (1 Jan 00 - 31 Dec 00)

4. TITLE AND SUBTITLE
Regulation of Cell Survival in Human Breast Cancer Cells by Sphingosine Kinase

5. FUNDING NUMBERS
DAMD17-99-1-9188

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

EDG-1 is a GPCR for sphingosine-1-phosphate (SPP) that mediates SPP-directed cell migration and vascular maturation. Cell migration toward PDGF, which stimulates sphingosine kinase and increases SPP levels, was dependent on expression of EDG-1, and conversely, deletion of EDG-1, inhibition of sphingosine kinase or treatment with pertussis toxin to uncouple Gi-linked receptors suppressed chemotaxis toward PDGF. PDGF activated EDG-1 in a sphingosine kinase-dependent manner as shown by translocation of B-arrestin. PDGF induced tyrosine phosphorylation of focal adhesion proteins, including FAK and Src, activation of the small GTPase Rac, essential for the protrusion of lamellipodia and forward movement, and p38 were nearly eliminated by the deletion of EDG-1, whereas tyrosine phosphorylation of the PDGFR and ERK activation were unaltered. Our results establish a new paradigm for receptor cross-communication in which transactivation of a GPCR, EDG-1, by a receptor tyrosine kinase, the PDGFR, is critical for cell motility.

14. SUBJECT TERMS

15. NUMBER OF PAGES
56

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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INTRODUCTION

The sphingolipid metabolite, sphingosine-1-phosphate (SPP), has emerged as a novel lipid second messenger involved in the regulation of stress responses, proliferation and survival of cells. SPP is produced after phosphorylation of sphingosine by sphingosine kinase, and has been shown to prevent apoptosis. Interest in SPP has accelerated recently with the discovery that it is the extracellular ligand for EDG-1, -3, -5, -6, and –8. Although the biological functions of these receptors have not been completely elucidated, we have recently implicated EDG-1 in cell migration (Wang et al,1999) and angiogenesis (Liu et al., 2000). .

Angiogenesis, the process of new vessel formation from pre-existing ones, or neovascularization, is essential throughout life, particularly during development, and is also important in certain pathological conditions such as tumor growth, invasion and metastasis. Recently, In collaboration with Dr. Proia's lab from the NIH we found that disruption of the *edg-1* gene by homologous recombination in mice resulted in massive intra-embryonic hemorrhaging and intrauterine death between E12.5 and E14.5 due to incomplete vascular maturation (Liu et al., 2000). This defect resulted from a failure of mural cells - vascular smooth muscle cells and pericytes - to migrate to arteries and capillaries and properly reinforce them, resulting in blood vessels composed of mainly naked endothelial tubes. Interestingly, disruption of the PDGF-BB or PDGFR-_ genes in mice resulted in a similar lethal phenotype. Since in many different cell types, PDGF stimulates sphingosine kinase leading to an increase in SPP levels, we hypothesized

that one of the critical mechanisms in PDGF-induced migration is transactivation of EDG-1, and that EDG-1 is necessary for PDGF-induced cell motility.

BODY

Overexpression of Sphingosine Kinase-1 Promotes Growth and Survival of Human Breast Cancer MCF-7 Cells:

Recently, Dr. Olivera in our lab demonstrated that sphingosine kinase-1 (SPHK-1) promotes cell growth and survival in non-transformed NIH 3T3 fibroblasts (J Cell Biol. 1999; 147(3): 545-58). Studying various human breast cancer cell lines did not reveal a correlation between levels of SPHK1 and cell growth or invasive phenotype. MCF-7 human breast cancer (HBC) cells were utilized for subsequent studies as they are readily transfectable and our lab has extensive experience with this cell line. Several MCF-7 clones stably expressing SPHK1 have been generated and I am now examining whether overexpression of SPHK-1 contributes to tumorigenesis of breast cancer MCF-7 cells. Overexpression of SPHK-1 in MCF-7 cells reduced cell death induced by the anti-cancer drug doxorubricin, sphingosine and TNF-α, all known to elevate levels of ceramide, another sphingolipid metabolite implicated in apoptosis. Furthermore, MCF-7 cells overexpressing SPHK-1 are also more resistant to the cytotoxic effects of N,N-dimethylsphingosine, a specific inhibitor of sphingosine kinase. In addition, SPHK-1 confers a small growth advantage to MCF-7 cells as determined by growth in soft agar and by proliferation assays. Although no changes in estrogen receptor levels could be detected, estrogen antagonists eliminated this growth advantage. In future studies, it will be determined

whether overexpression of SPHK1 in MCF-7 cells results in larger subcutaneous tumors in ovariectomized nude mice implanted with 17B-estradiol pellets. These studies will support a role for SPHK-1 in tumor progression.

Mitogenic and Survival Responses of SPP are Independent of EDG-1 Expression:

As mentioned above, the mitogenic effect of SPP is one of its most well established biological responses. Our recent studies suggest that EDG-1 plays a critical role in proliferation of endothelial cells (Wang et al 1999). Thus, it was of interest to examine whether EDG-1 mediates the proliferative and survival effects of SPP. Fibroblasts isolated from EDG-1 knockout mouse embryos were used to determine whether these proliferative responses are regulated by binding of SPP to EDG-1. Unexpectedly, the mitogenic effect of SPP was not abrogated in EDG-1-/- fibroblasts (Fig. 1A). Moreover, sphingosine, which is readily taken up by cells and converted intracellularly to SPP, was equally as effective in stimulating proliferation of EDG-1 -/- fibroblasts as wild type fibroblasts. Similarly, the mitogenic effects of PDGF-BB and fetal bovine serum (FBS) were not significantly different in wild-type and EDG-1 -/- cells **(Fig. 1A)**. Surprisingly, disruption of the *edg-1* gene also had no significant effect on apoptosis and addition of SPP, to control or EDG-1 -/- fibroblasts markedly suppressed apoptosis induced by serum deprivation or the chemotherapeutic drug doxorubicin. These protective effects were specific and unrelated to EDG-1, because dihydro-SPP, which lacks the trans double bond present in SPP yet binds and activates EDG-1 equally well, did not significantly prevent apoptosis in wild-type or mutant fibroblasts **(Fig. 1B)**.

Transactivation of EDG-1 is Important for PDGF-Induced Cell Motility:

The next aim was to study the involvement of EDG-1 in SPP- and PDGF-induced motility. To this end, three cell types were used: human embryonic kidney cells (HEK 293), mouse embryonic fibroblasts (MEFs), and aortic smooth muscle cells (ASMC). HEK 293 cells, which only express EDG-3 and EDG-5, were used to generate overexpressing EDG-1 cell lines. These cells were then used in a Boyden chamber to quantify cell migration. HEK 293 cells did not migrate toward SPP unless EDG-1 was expressed. EDG-1 overexpression also stimulated migration of HEK 293 cells toward PDGF-BB, whereas migratory responses to serum and fibronectin were unaltered **(Fig. 2)**. To further substantiate the involvement of EDG-1, cells were pre-treated with pertussis toxin (PTX). PTX is a bacterial toxin that ADP-ribosylates the α-subunit of Gi coupled GPCR, thus inactivating signaling from the receptor. PTX suppressed both SPP- and PDGF-induced chemotaxis of HEK 293 cells overexpressing EDG-1 **(Fig. 3 B).**

It has been demonstrated that PDGF can activate SPHK leading to the formation of SPP. Since both SPP- and PDGF-induced motility were inhibited by PTX, it was my hypothesis that PDGF stimulated SPHK and subsequent formation of SPP leading to increased migratory responses. This was examined by treating cells with N,N-dimethylsphingosine (DMS), a potent competitive inhibitor of SPHK. When the cells were pretreated with DMS, PDGF-induced migration was inhibited **(Fig. 3B)**.

As the vascular phenotypes of EDG-1, PDGF-BB and PDGFR-B null mice are similar, it was of importance to examine the effect of *edg-1* deletion on cell migratory responses to PDGF.

In striking contrast to wild-type fibroblasts, *edg-1* deleted fibroblasts showed markedly reduced migration toward PDGF-BB. A smaller effect on migration toward serum was observed in these mutant fibroblasts and migration toward fibronectin was unaffected **(Fig. 4).** These results suggest that *edg-1* deletion does not disrupt all essential mechanisms of directed cell movement. Additionally, PTX and DMS inhibited PDGF induced migration of wild-type MEFs **(Fig. 3 A)**.

Because the migration of smooth muscle cells appears to be aberrant in EDG-1 knockout mice, the role of EDG-1 in PDGF-directed migration of ASMC was also examined by treatment with EDG-1 antisense oligonucleotides designed to bind to the translational initiation site on the EDG-1 mRNA and reduce its expression. Treatment of ASMC, which similar to MEFs, endogenously express EDG-1, -3, and -5, with EDG-1 antisense phosphothioate oligonucleotide, not only eliminated migration toward SPP but also significantly reduced migration toward PDGF-BB, but not serum **(Fig. 5)**. These results suggest that the loss of EDG-1 results in motility defects toward PDGF in diverse cell types.

Because EDG-1 was found to be required for PDGF-induced motility and it has previously been demonstrated that PDGF can activate SPHK to form SPP, It was possible that stimulation with PDGF could lead to EDG-1 activation. When EDG-1 is activated by its ligand, it is phosphorylated at the third intracellular loop by G-protein coupled receptor kinases. Thus, the phosphorylation status of EDG-1 as a marker for its activation. To enhance sensitivity of detection, HEK 293 cells overexpressing Flag epitope-tagged EDG-1 were transfected with an expression plasmid encoding PDGFR, labeled in situ with $^{32}P_i$, and EDG-1 was

immunoprecipitated with anti-Flag antibody. Similar to SPP, PDGF increased phosphorylation of EDG-1 in these cells, whereas no phosphorylation could be detected in vector transfected cells **(Fig. 6)**. This result indicates that PDGF stimulation induces SPP production that can activate EDG-1.

KEY RESEARCH ACCOMPLISHMENTS

- Overexpression of SPHK-1 in MCF-7 cells reduced cell death induced by anti-cancer drugs, sphingosine, and TNF-α
- Overexpression of SPHK-1 confers a small growth advantage to MCF-7 cells as determined by growth in soft agar and by proliferation assays
- Demonstrated that EDG-1 is not required for cell growth or cell survival.

- Accumulated abundant evidence demonstrating that SPP induces migration via EDG-1.
- Demonstrated a potential role for EDG-1 in PDGF-induced motility by using an antisense approach, EDG-1 null fibroblasts, and EDG-1 overexpressing cells.
- Demonstrated that PDGF-induced motility is both PTX and DMS sensitive; implicating roles for the Gi coupled receptor EDG-1 and sphingosine kinase.
- Proposed the novel concept of crosstalk between receptor tyrosine kinases and GPCRs in which transactivation of EDG-1 by PDGF through the activation of SPHK leading to formation of SPP and subsequent activation of EDG-1 plays a critical role in cell motility.

CONCLUSIONS

Cross communication between receptor signaling cascades enhances the ability of cells to respond appropriately to different stimuli. Recently, several reports have shown that various agonists for GPCRs can activate growth factor tyrosine kinase receptors in the absence of added growth factors. While this type of transmembrane cross communication is important for regulation of cell growth, my results suggest that cell motility is regulated by a reciprocal

mechanism of receptor crosstalk. Thus, stimulation of PDGFR by PDGF activates sphingosine kinase resulting in increased formation of SPP. SPP in turn, in an autocrine or paracrine fashion, stimulates EDG-1 which is critical for cell locomotion.

These observations have important physiological relevance for new blood vessel formation and microvasculature maturation. During blood vessel formation, B-PDGFR positive pericytes and smooth muscle cells are recruited to the vessel walls through the action of PDGF-BB secreted from endothelial cells. Dysfunctional migration of EDG-1 -/- cells toward PDGF links this phenotype to the PDGF-BB and B-PDGFR knockout phenotypes at the final steps of vasculogenesis underscoring the importance of cross-talk between PDGFR and EDG-1 in vascular maturation. These findings are highly pertinent to cancer cell growth and metastasis as it reveals a new regulator of blood vessel maturation and is a fruitful area for further investigations in breast cancer research.

Future investigations will examine the signaling molecules involved in PDGF-induced cell migration that are regulated by transactivation of EDG-1. I will attempt to elucidate roles for FAK, Src, and other molecules that may be involved in EDG-1 regulated migration. Elucidation of the molecular mechanisms by which sphingosine kinase regulates vascular maturation might provide clues for development of a new class of therapeutic agents to block mature neovessel formation through effects on SPP/EDG-1 signaling.

Revised Statement of Work: The original proposal was focused on the role of sphingosine kinase only in cell growth and survival of human breast cancer (HBC) cells. However, progress toward achievement of Technical Objective 1 did not reveal a correlation between level of sphingosine kinase and cell growth. Moreover, my results indicate that EDG-1, the downstream target of sphingosine kinase, is not required for cell growth or survival, but rather for cell motility. Thus, in addition to focusing on cell growth and apoptosis, I would also like to focus on cell migration. Technical Objectives 1 and 2 remain unchanged but a modification to Technical Objective 3 is requested.

Technical Objective 1: Characterization of human breast cancer (HBC) cell lines to determine base line levels of SPHK expression, SPHK activity, levels of SPP, and sensitivity to apoptotic stimuli, such as TNF-α ionizing radiation, and chemotherapeutics, known to elevate ceramide levels.

There are no changes in this objective which is already well under way.

Technical Objective 2: Generation of HBC cell lines that stably overexpress SPHK from an inducible vector and the evaluation of the effects of overexpression on susceptibility to apoptosis.

There are no changes in this objective which is already well under way.

New Technical Objective 3: Examination of the role of downstream target of sphingosine kinase, EDG-1, in cell migration.

Task 1: Months 19-22: Examining the role of the small GTPases in sphingosine kinase-regulated cell migration.

Task 2: Months 23-26: Generating cell lines overexpressing dominant negative small GTPases to further substantiate their role in sphingosine kinase-regulated cell migration.

Task 3: Months 26-30: Examining the role of the cytosolic tyrosine kinases Src and focal adhesion kinase in sphingosine kinase-regulated cell migration.

Task 4: Months 30+: Generating cell lines overexpressing dominant negative Src and FAK to further substantiate their role in sphingosine kinase-regulated cell migration. Completion of all other studies.

REPORTABLE OUTCOMES

PAPERS

Wang, F., Van Brocklyn, J.R., Van Brocklyn JR, **Hobson JP**, Movafagh,S., Zukowska-Grojec, Z., Milstien S., and Spiegel, S. (1999) Sphingosine-1-phosphate stimulates chemotaxis through a G_i-coupled cell surface receptor: Potential involvement in angiogenesis. *J. Biol. Chem.* 274: 35343-3550,

Liu, Y., Wada, R., Yamashita, T., Deng, C.X., **Hobson JP** Rosenfeldt, H.M., Nava, V.E., Van Brocklyn, J.R., Liu, C.H., Hla, T., Spiegel, S., and Proia, R.L. (2000)The G-protein coupled sphingosine-1-phosphate receptor, EDG-1, is essential for vascular morphogenesis. *J. Clin. Invest.* 106: 951-961.

Hobson JP, Rosenfeldt HM, Barak LS, Olivera A, Poulton S, Caron MG, Milstien S, and Spiegel S. (2001) Role of the Sphingosine-1-Phosphate Receptor EDG-1 in PDGF-induced Cell Motility. Science, In Press.

ABSTACTS

John P. Hobson, Fang Wang, James R. Van Brocklyn, Sheldon Milstien and Sarah Spiegel. Involvement of Sphingosine-1-Phosphate in Angiogenesis SEBM Graduate Student Research Forum. April 6, 2000. Washington, D.C.

John P. Hobson, Fang Wang, James R. Van Brocklyn, Sheldon Milstien and Sarah Spiegel. Involvement of Sphingosine-1-Phosphate in Angiogenesis. Keystone Symposium on Experimental and Clinical Regulation of Angiogenesis that was held in Salt Lake City, UT on March 2-7, 2000.

PRESENTATIONS

Hobson JP, Binding of Sphingosine-1-Phosphate to Edg Plays a Role in Regulating Cell Migration. November 21, 2000. Georgetown University, Department of Biochemistry.

INVOLVEMENT OF SPHINGOSINE-1-PHOSPHATE IN ANGIOGENESIS

John P. Hobson, Fang Wang, James R. Van Brocklyn, Sheldon Milstien and Sarah Spiegel. Georgetown University Medical Center, Department of Biochemistry and Molecular Biology, Washington D.C. 20007.

Sphingosine-1-phosphate (SPP), a sphingolipid metabolite, is a bioactive lipid second messenger implicated in cell survival and proliferation. SPP also inhibits chemotaxis of many cells, in some cases through intracellular actions, while in others through receptor-mediated effects. Surprisingly, we found that low concentrations of SPP (10-100 nM) increased chemotaxis of HEK293 cells over expressing the G protein-coupled SPP receptor EDG-1, while at μM concentrations, SPP inhibited chemotaxis of both vector transfected and HEK293-EDG-1 cells. Nanomolar concentrations of SPP also induced a marked increase in chemotaxis of bovine aortic endothelial cells (BAEC), which express the SPP receptors EDG-1 and to a lesser extent EDG-3. Treatment with pertussis toxin, which inactivates G_I-coupled receptors by ADP-ribosylation of the G_i subunit, blocked SPP-induced chemotaxis. Sphinganine-1-phosphate (dihydro-SPP), which also binds to SPP receptors, enhanced chemotaxis; whereas lysophosphatidic acid, did not compete with SPP for binding nor did it have significant effects on chemotaxis of endothelial cells. Furthermore, SPP increased proliferation of BAE cells in a pertussis toxin-sensitive manner. SPP and dihydro-SPP stimulated tube formation of BAE cells grown on collagen gels (*in vitro* angiogenesis) was also inhibited by pertussis toxin. As expected pertussis toxin did not inhibit tube formation stimulated by bFGF, a potent angiogenic factor of endothelial cells. These results combined with recent studies employing antisense oligonucleotides directed against the EDG-1 mRNA (Lee et al., 1999) suggest that SPP binding to the EDG-1 receptor plays an important role in angiogenesis.

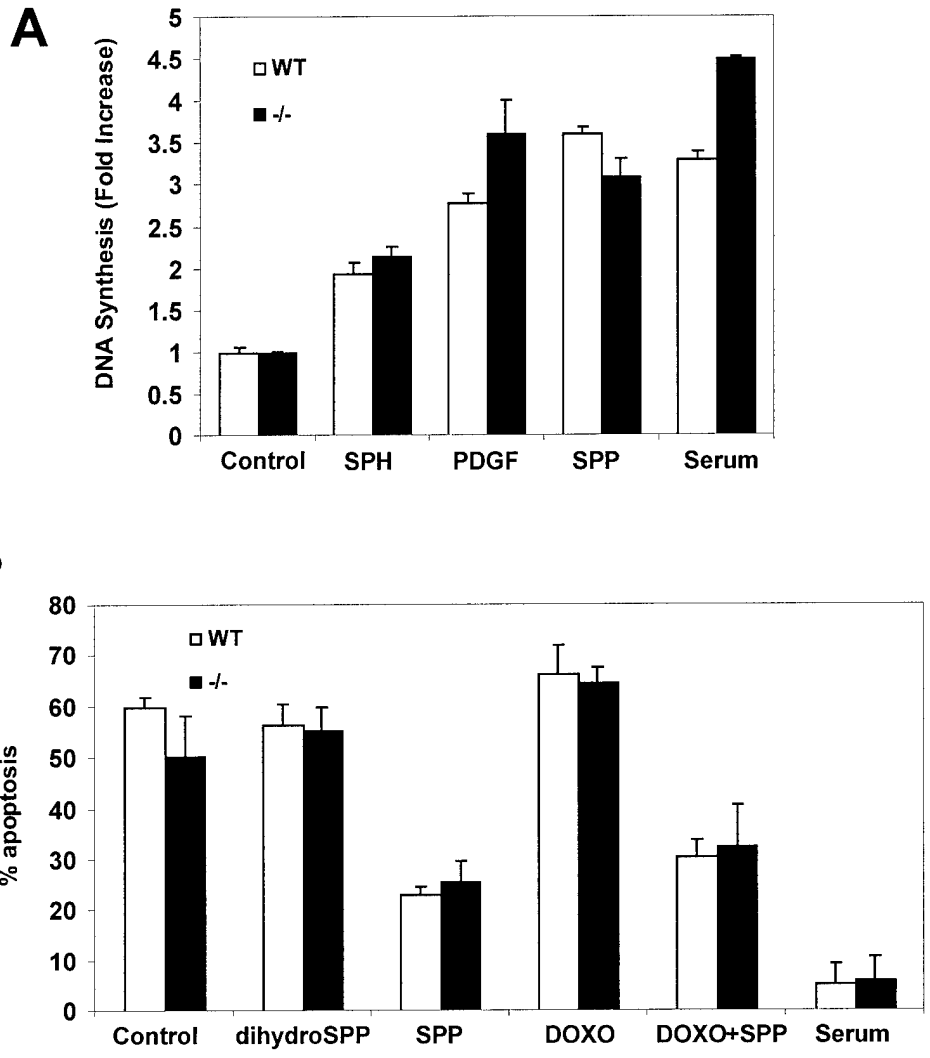
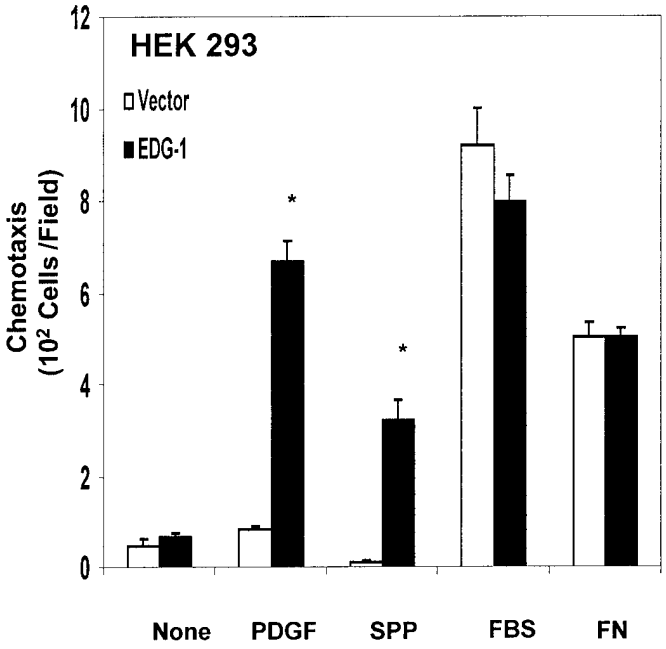


Figure 1. EDG-1 is not required for SPP-stimulated proliferation and survival. **(A)** Confluent and quiescent wild-type (open bars) and EDG-1 -/- mouse embryonic fibroblasts (filled bars) were treated with PDGF (20 ng/ml), serum (10 % v/v), sphingosine (SPH), or SPP (10 μM) and DNA synthesis was measured by [^{3}H]thymidine incorporation [Van Brocklyn, 1998 #2303]. Data are expressed as fold stimulation and are means ± S.D. from three independent experiments, each analyzed in triplicate. **(B)** Subconfluent wild-type (open bars) and EDG-1 -/- mouse embryonic fibroblasts (filled bars) were cultured in serum-free medium for 48 h, in the absence (Control) or presence of serum (10%), SPP (10 μM), dihydro-SPP (10 μM), or doxorubicin (DOXO, 1 μg/ml) in the absence and presence of SPP (10 μM), and apoptosis determined. Data are means ± S.E. of four independent experiments, each analyzed in triplicate.

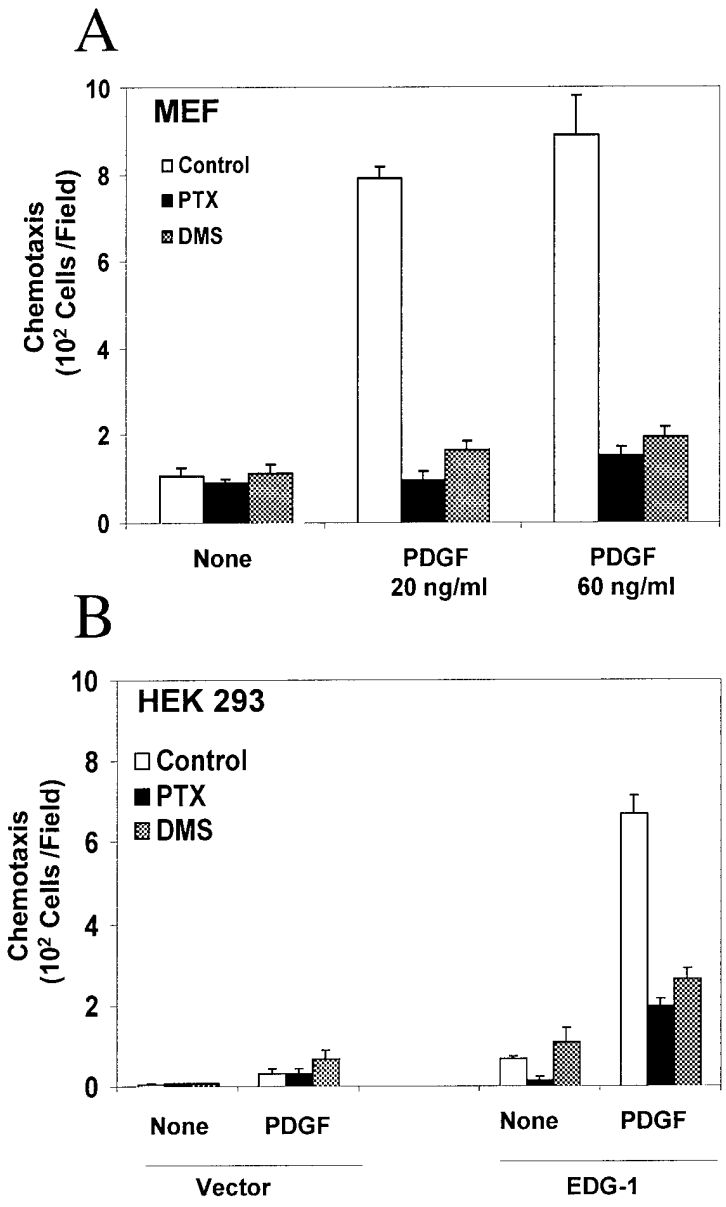
Figure 1

FIG 2



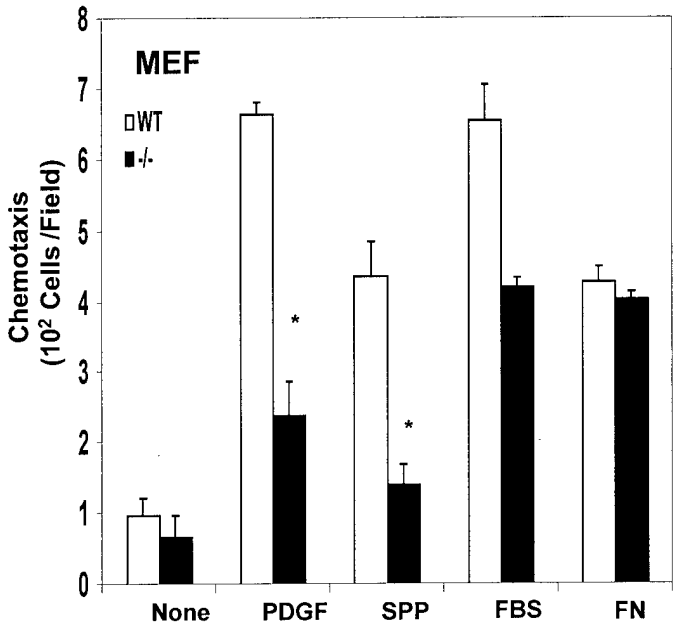
Requirement of EDG-1 for PDGF-induced migration. Enhanced chemotaxis toward PDGF in cells overexpressing EDG-1. HEK 293 cells stably transfected with vector (open bars) or EDG-1 (filled bars) were allowed to migrate toward PDGF-BB (20 ng/ml), SPP (100 nM), serum (FBS, 20 %), or fibronectin (FN, 10 μg/ml). Chemotaxis was measured in a modified Boyden chamber assay . The average number of cells in four random fields was determined and is presented as the average ± S.D. of three individual wells. Similar results were obtained in at least three independent experiments and statistically different groups are indicated by asterisk ($p < 0.05$ by analysis of variance)

FIG 3



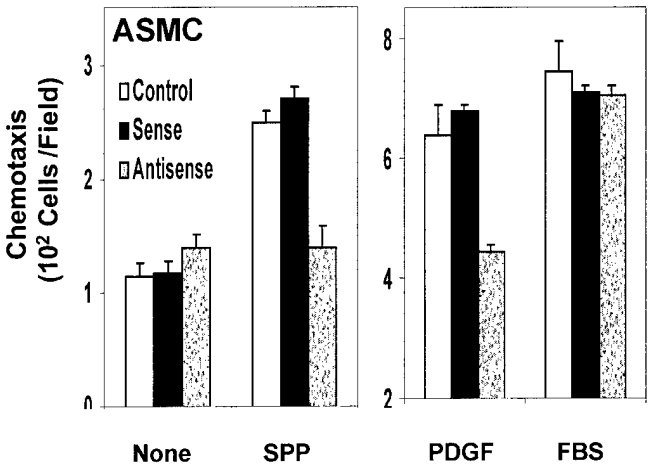
Inhibition of chemotaxis toward PDGF by the sphingosine kinase inhibitor DMS or pertussis toxin. Wild-type MEFs **(Top Panel)** or HEK 293 cells transfected with vector or EDG-1 **(Bottom Panel)** were treated without (Control) or with pertussis toxin (PTX, 200 ng/ml, 2 hours), or DMS (10 μM, 20 min), then allowed to migrate towards the indicated concentrations of PDGF-BB and chemotaxis was measured using a Boyden chamber.

FIG 4



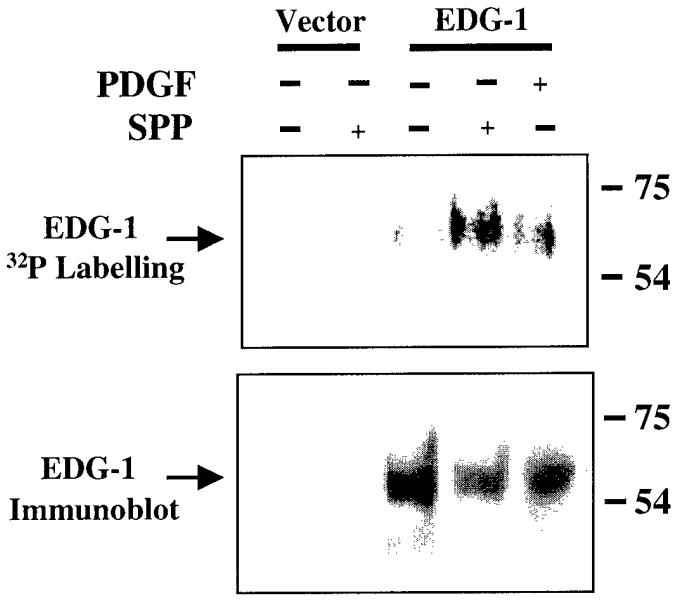
Chemotaxis toward PDGF is markedly reduced by edg-1 disruption. Wild type (open bars) and EDG-1 -/- (filled bars) MEFs (Mouse Embryonic Fibroblasts) were allowed to migrate toward PDGF-BB, SPP, serum or FN as in Fig. 1.

FIG 5



SPP and PDGF-stimulated chemotaxis of human aortic smooth muscle cells is dependent on EDG-1 expression. ASMCs were transfected without (open bars) or with 400 nM EDG-1 sense (filled bars) or antisense (gray bars) PTOs and after 24 h, allowed to migrate toward SPP (100 nM), PDGF-BB (20 ng/ml), or serum (20%). Similar results were obtained in two independent experiments.

FIG 6



PDGF-induced EDG-1 phosphorylation. Vector or Flag-EDG-1-expressing HEK 293 cells were transfected with PDGFR- and cultured in 10% charcoal-stripped FBS for 24 hours, metabolically labeled in phosphate free DMEM with [^{32}P]orthophosphate (70 μCi/ml) for 2.5 hours at 37 °C, then stimulated with SPP (100 nM) or PDGF (20 ng/ml). Cell lysates were prepared and immunoprecipitated with anti-Flag M2 antibody (Sigma). Immunoprecipitates were separated on 10% SDS-PAGE, transblotted to nitrocellulose, and autoradiographed (upper panel) or immunoblotted with anti-Flag antibody (lower panel).

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 274, No. 50, Issue of December 10, pp. 35343–35350, 1999
Printed in U.S.A.

Sphingosine 1-Phosphate Stimulates Cell Migration through a G_i-coupled Cell Surface Receptor

POTENTIAL INVOLVEMENT IN ANGIOGENESIS*

(Received for publication, April 22, 1999, and in revised form, August 20, 1999)

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Sphingosine 1-phosphate (SPP) has been shown to inhibit chemotaxis of a variety of cells, in some cases through intracellular actions, while in others through receptor-mediated effects. Surprisingly, we found that low concentrations of SPP (10–100 nM) increased chemotaxis of HEK293 cells overexpressing the G protein-coupled SPP receptor EDG-1. In agreement with previous findings in human breast cancer cells (Wang, F., Nohara, K., Olivera, O., Thompson, E. W., and Spiegel, S. (1999) *Exp. Cell Res.* 247, 17–28), SPP, at micromolar concentrations, inhibited chemotaxis of both vector- and EDG-1-overexpressing HEK293 cells. Nanomolar concentrations of SPP also induced a marked increase in chemotaxis of human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC), which express the SPP receptors EDG-1 and EDG-3, while higher concentrations of SPP were less effective. Treatment with pertussis toxin, which ADP-ribosylates and inactivates G_i-coupled receptors, blocked SPP-induced chemotaxis. Checkerboard analysis indicated that SPP stimulates both chemotaxis and chemokinesis. Taken together, these data suggest that SPP stimulates cell migration by binding to EDG-1. Similar to SPP, sphinganine 1-phosphate (dihydro-SPP), which also binds to this family of SPP receptors, enhanced chemotaxis; whereas, another structurally related lysophospholipid, lysophosphatidic acid, did not compete with SPP for binding nor did it have significant effects on chemotaxis of endothelial cells. Furthermore, SPP increased proliferation of HUVEC and BAEC in a pertussis toxin-sensitive manner. SPP and dihydro-SPP also stimulated tube formation of BAEC grown on collagen gels (*in vitro* angiogenesis), and potentiated tube formation induced by basic fibroblast growth factor. Pertussis toxin treatment blocked SPP-, but not bFGF-stimulated *in vitro* angiogenesis. Our results suggest that SPP may play a role in angiogenesis through binding to endothelial cell G_i-coupled SPP receptors.

The sphingolipid metabolite sphingosine 1-phosphate (SPP)[1] is a bioactive lipid that regulates diverse biological effects and signaling pathways (reviewed in Ref. 1). SPP increases cell proliferation (2, 3) and opposes ceramide-mediated apoptosis (4–7) through an intracellular action (8, 9), yet some of its biological effects when added exogenously are due to binding to cell surface receptors. Pertussis toxin-sensitive G proteins are involved in some of the signaling pathways activated by SPP (10–15), suggesting that it activates a receptor coupled to a G_i/G_o protein. In agreement, low concentrations of SPP activate G_i protein-gated inward rectifying K^+ channels only when applied at the extracellular face of atrial myocytes (16).

Several reports have demonstrated that SPP inhibits cell motility. SPP inhibited chemotactic motility of mouse melanoma B16, mouse fibroblast BALB/3T3 clone A31, and several tumor cell lines at nanomolar concentrations (17–19). Moreover, SPP immobilized on glass beads markedly inhibited melanoma cell motility. However, pertussis toxin treatment did not block the effect of SPP, suggesting that in these cells SPP acts through a cell surface receptor, independently of pertussis toxin-sensitive G-proteins (20). In contrast, SPP inhibits chemotaxis of human breast cancer cells only at high (micromolar) concentrations, acting independently of EDG-1 (21).

We have recently identified SPP as a ligand for the G-protein-coupled receptor, endothelial differentiation gene-1 (EDG-1) (22). EDG-1 binds SPP with remarkable specificity and high affinity (K_D = 8 nM) (9, 22). Binding of SPP to EDG-1 resulted in inhibition of adenylate cyclase and activation of mitogen-activated protein kinase (both G_i-mediated), but did not mobilize calcium from internal stores (9, 23). In contrast, Okamoto *et al.* (12) found that in HEL cells overexpressing EDG-1, binding of SPP induced calcium mobilization (12).

Two other related G protein-coupled receptors, EDG-3 and EDG-5, have recently been shown to bind SPP with similar high affinity (14, 24), to confer responsiveness to SPP of a serum response element-driven reporter gene when expressed in Jurkat cells, and to allow SPP-stimulated $^{45}Ca^{2+}$ efflux in *Xenopus* oocytes (25). In agreement, overexpression of EDG-3 in Chinese hamster ovary cells led to phospholipase C activation and calcium mobilization induced by SPP, which was significantly inhibited by pertussis toxin (26). However, low con-

* This work was supported in part by National Institutes of Health Research Grant CA61774 (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Predoctoral Fellowship BC961968 from the United States Army Medical Research and Material Command, Breast Cancer Research Program.

§ Supported by Postdoctoral Fellowship F32 GM19209 from the National Institutes of Health.

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[1] The abbreviations used are: SPP, sphingosine 1-phosphate; BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cells; GPCR, G protein-coupled receptors; dihydro-SPP, sphinganine 1-phosphate; LPA, lysophosphatidic acid; IMEM, Richter's improved minimal essential medium; bFGF, basic fibroblast growth factor; EDG-1, endothelial differentiation gene-1; SPC, sphingosylphosphorylcholine; RT-PCR, reverse transcriptase-polymerase chain reaction.

centrations of SPP mobilize calcium from internal sources in BAEC in a pertussis toxin-sensitive manner without activation of phospholipase C (27), suggesting the involvement of novel, unidentified signaling pathways in SPP-induced release of intracellular calcium.

Although the biological functions of the EDG family of GPCR are not completely understood, the EDG-1 transcript was originally cloned as an immediate-early gene induced during differentiation of HUVEC, cells of the vessel wall accessible to platelet-derived ligands, into capillary-like tubules (28). Moreover, SPP signaling in HEK293 cells overexpressing EDG-1 leads, by a Rho-dependent mechanism, to formation of a network of cell-cell aggregates resembling the network formation of differentiated endothelial cells and P-cadherin expression (22). Because SPP is stored and released from activated platelets and serum concentrations of SPP are estimated to be approximately 0.5 μM (29), about 60 times greater than the K_D for binding to EDG-1, we suggested that SPP might play an important role in angiogenesis acting through EDG-1 (22).

Angiogenesis, the process of new vessel formation from preexisting ones, or neovascularization, is a critical event for a variety of physiological processes, such as wound healing, embryonic development, corpus luteum formation, and menstruation. However, angiogenesis can be activated in response to tissue damage and is important in certain pathological conditions such as tumor growth and metastasis, rheumatoid arthritis, diabetic retinopathy, psoriasis, and cardiovascular diseases (30). Conversely, in states of inadequate tissue perfusion, such as myocardial or limb ischemia, enhanced angiogenesis is essential and beneficial (31). Endothelial cell migration and formation of new capillary tubes are required events in the angiogenic response. In this study, we investigated the potential role of SPP in angiogenesis by examining regulation of endothelial cell motility, proliferation, and tube formation through SPP receptors.

EXPERIMENTAL PROCEDURES

Materials—IMEM, penicillin/streptomycin, L-glutamine, amphotericin B, fetal calf serum, and fetal bovine serum were from Biofluids (Rockville, MD). Medium 199 was from Life Technologies (Gaithersburg, MD). Calf serum was from Colorado Serum Co. (Denver, CO), and Matrigel, bFGF, endothelial cell growth supplement, and rat tail type I collagen were from Collaborative Biomedical Products (Bedford, MA). SPP, dihydro-SPP, and sphingosylphosphorylcholine (SPC) were obtained from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (LPA) was from Avanti Polar Lipids, Inc. (Alabaster, AB). The Diff-Quik kits were from Sigma. [*methyl*-^{3}H]Thymidine (55 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

Cell Culture—Human embryonic kidney cells (HEK293, ATCC CRL-1573) and HEK293-*EDG-1* cells, kindly provided by Drs. Menq-Jer Lee and Timothy Hla, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, with 1 mg/ml G418 sulfate for HEK293-*EDG-1*, as described previously (9, 22). BAEC were kindly provided by Dr. Luyuan Li and maintained in IMEM containing 10% fetal bovine serum supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 1 ng/ml bFGF. HUVECs were isolated as previously reported (32), and grown in medium 199 supplemented with gentamycin, 2 mM glutamine, 500 units/dl sodium heparin, 2.5 mg/dl amphotericin B, and 2 mg/dl endothelial cell growth supplement.

SPP Binding Assay—HUVEC or BAEC were washed with binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 15 mM sodium fluoride, 2 mM deoxypyridoxine, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin) and removed from dishes by scraping. Cells were then pelleted and resuspended in binding buffer containing 4 mg/ml BSA. 10^5 cells were incubated with 0.2 nM [^{32}P]SPP, synthesized enzymatically using recombinant sphingosine kinase (33) as described previously (9), in 0.2 ml of binding buffer plus 4 mg/ml BSA for 30 min at 4 °C in the absence or presence of 1000-fold excess unlabeled SPP or other lipid competitors, added as 4 mg/ml fatty acid-free BSA complexes. Cells were then pelleted at 8,000 rpm in a microcentrifuge, washed twice with binding buffer containing 0.4 mg/ml fatty acid-free BSA, resuspended in binding buffer without BSA and bound [^{32}P]SPP quantitated by scintillation counting. The phosphatase and protease inhibitors were included in the binding assays as a precaution against the possibility that cells which may have been damaged during scraping might leak phosphatases or proteases which could cleave SPP or EDG receptors, respectively. In addition, it has been proposed that cell surface lipid phosphatases which can cleave exogenous SPP exist (34). Nevertheless, identical specific binding of [^{32}P]SPP was obtained in the absence of the protease and phosphatase inhibitors. It should be pointed out that SPP is not metabolized during the binding assay. When [^{32}P]SPP was incubated in the absence or presence of endothelial cells under the same conditions as the binding assay, no decrease in the amount of [^{32}P]SPP was detected by TLC nor did any additional bands appear.

Reverse Transcriptase (RT)-PCR—The cDNA encoding the open reading frame of EDG-1 was amplified with the Gene Amp RNA-PCR kit (Perkin-Elmer) using RNA isolated with TRIzol Reagent (Life Technologies) and digested with RNase-free DNase I (RQ-1, Promega). The primers (Life Technologies) used for PCR amplification were 5′-GATATCATCGTCCGGCATTAC and 5′-ACCCTTCCCAGTGCATTGTTC for *EDG-1* (28); 5′-CACTCAGCAATGTACCTGTTCC and 5′-AACACCCAGTACGATGGTGAC for *EDG-5* (35, 36); and 5′-GACTGCTCTACCATCTTGCCC and 5′-GTAGATGACAGGGTTCATGGC for *EDG-3* (37). PCR reactions were performed for 30 cycles with denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 50 s. PCR products were analyzed by agarose gel electrophoresis after staining with ethidium bromide.

Migration—Chemotactic migration of cells in response to a gradient of SPP was measured in a modified Boyden chamber as described previously (21). In brief, polycarbonate filters (5 μm for BAEC and 8 μm for HUVEC) were coated with gelatin (0.1%) overnight. Cells were harvested by trypsinization, washed with serum-free IMEM containing 0.1% fatty acid-free BSA, and were added to the upper wells (24-multiwell Boyden microchambers) at 1×10^5 cells per well; the lower wells contained SPP diluted in serum-free IMEM containing 0.1% BSA. After 2 h at 37 °C in 5% CO_2, non-migratory cells on the upper membrane surface were removed with a cotton swab and the cells which traversed and spread on the lower surface of the filter were fixed and stained with Diff-Quik. The number of migratory cells per membrane was enumerated using a microscope with a ×20 objective. Each data point is the average number of cells in four random fields, each counted twice. Each determination represents the average ± S.D. of three individual wells. Checkerboard assays were carried out as described above except that various dilutions of SPP in fatty acid-free BSA were placed in the top and/or bottom wells of the Boyden chamber. In most of the experiments, unless indicated otherwise, cells were serum starved for 2 h prior to the assays.

[^{3}H]Thymidine Incorporation Assays—Cells were seeded at an initial density of 5×10^4 cells per well in 24-well plates and allowed to attach overnight. Confluent BAEC were growth arrested in culture media without bFGF for 48 h and then treated for 24 h with SPP or bFGF. Since the sensitivity of BAEC to stimulation by bFGF and SPP decreased with passage number, all experiments were carried out with cells at less than passage 12. Confluent HUVEC were serum-starved for 24 h and then treated with different concentration of SPP in medium 199 containing 1% FCS and 1000 units/dl heparin for 24 h. [^{3}H]Thymidine (1 μCi/ml) was added 8 h before termination of the assay, and [^{3}H]thymidine incorporation into DNA was measured as described (38). Values are the means of triplicate determinations and standard deviations were routinely less than 10% of the mean.

In Vitro Angiogenesis—Three-dimensional collagen gel plates (24 well) were prepared by addition of 0.5 ml of a chilled solution of 0.7 mg/ml rat tail type I collagen in Dulbecco's modified Eagle's medium adjusted to neutral pH with $NaHCO_3$. After formation of the collagen gel (about 1–2 mm thickness), BAEC were seeded at 50,000 cells/well. At 80% confluency, culture medium was changed to media without bFGF and incubation was continued for 48 h, by which time the cells formed a monolayer on the gel. The cells were then treated with different concentrations of bFGF or SPP as indicated. Cultures were maintained at 37 °C for 48 h and then fixed with cold methanol. The gels were then soaked in phosphate-buffered saline/glycerol (1:1) and transferred onto glass slides. The extent of tube-like structures that formed in the gel was measured as total length per field using computer-assisted imaging with a Hamamatsu C2400 video camera and a Zeiss Axioscope microscope to quantitate the extent of tube formation. Three culture wells were used for each sample, and three microscopic fields

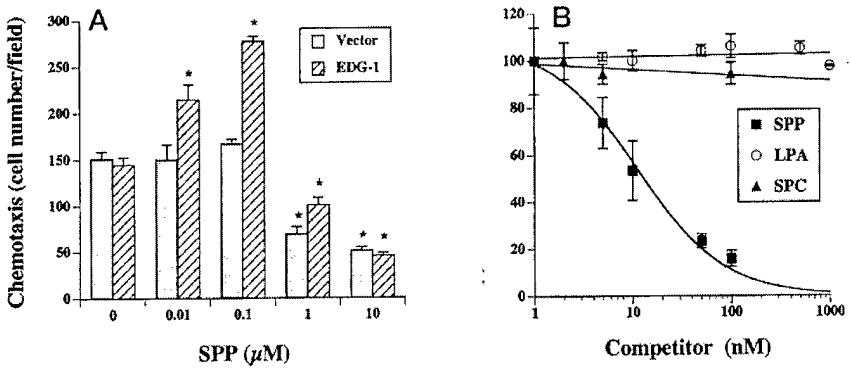

FIG. 1. **Effect of SPP on chemotaxis of HEK293 cells overexpressing EDG-1.** *A*, stable vector-transfected (*closed bars*) or *EDG-1*-transfected (*hatched bars*) HEK293 cells were treated with the indicated concentrations of SPP, and chemotaxis was measured as described under "Experimental Procedures." Data are mean ± S.D. of triplicate determinations. Similar results were obtained in three independent experiments. The *asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$) compared with untreated cells. *B*, competition by Lysosphingolipids for [^{32}P]SPP binding to HEK293 cells overexpressing EDG-1. HEK293-*EDG-1* cells were incubated with 0.2 nM [^{32}P]SPP in the absence or presence of the indicated concentrations of unlabeled SPP, LPA, or SPC and specific binding was measured as described under "Experimental Procedures."

were examined for each well. Thus, each experimental point represents results from examination of nine microscopic fields.

RESULTS

Effect of SPP on Chemotaxis of EDG-1-overexpressing Cells—SPP was previously shown to inhibit chemotaxis of mouse melanoma cells by binding to a putative cell surface receptor (20). Since we have recently identified EDG-1 as a receptor for SPP (22), it was of interest to examine the involvement of EDG-1 in SPP-regulated cell motility. Human embryonic kidney 293 fibroblasts stably expressing EDG-1 (HEK293-*EDG-1*) were selected for these studies since they have high levels of specific SPP binding, whereas SPP binding is nearly undetectable to parental and vector transfected cells (9, 22). Surprisingly, low concentrations of SPP (10–100 nM) did not inhibit, but rather enhanced chemotaxis of HEK293-*EDG-1* cells by 1.5–1.9-fold, while chemotaxis of vector-transfected cells was not altered by these concentrations of SPP (Fig. 1*A*). The concentrations of SPP which stimulate chemotaxis of HEK293-*EDG-1* cells are in the same range as the measured affinity of EDG-1 for SPP (K_D = 8 nM) (22) and correlate closely with binding and inhibition of forskolin-stimulated cAMP accumulation in these cells (9). These results suggest that low concentrations of SPP may increase chemotaxis by binding to EDG-1. Higher concentration of SPP (1–10 μM), as previously reported in human breast cancer cells (21), inhibited, rather than stimulated, chemotaxis of both vector and *EDG-1*-transfected cells.

In contrast to undetectable levels of *EDG-1* mRNA in parental and vector transfected HEK293 cells (9, 39), HEK293-*EDG*-1 cells express very high levels of *EDG*-1 mRNA as detected by Northern analysis (9) and RT-PCR (Fig. 2*A*). Additionally, a low level of *EDG-3* mRNA and *EDG-5* mRNA were detected by RT-PCR in these cells (Fig. 2*A*). Similar to our previous results (9), both unlabeled SPP and dihydro-SPP effectively competed with [^{32}P]SPP for binding to EDG-1 (data not shown), whereas, LPA and SPC were completely ineffective (Fig. 1*B*). Moreover, LPA, even at concentrations as high as 10 μM, and for prolonged incubations, had no significant effect on SPP binding to HEK293-*EDG*-1 cells.

Expression of EDG Receptors in Endothelial Cells—Previously, it has been demonstrated that human endothelial cells express high levels of *EDG-1* mRNA (28). Thus, it was of interest to examine whether the other SPP receptors, EDG-3 and EDG-5 (24, 25, 40), are also expressed in HUVEC as well as BAEC which, based on indirect evidence, have been proposed to have putative G_i-coupled SPP receptors linked to calcium mobilization (27). Consistent with previous studies, RT-PCR analysis clearly demonstrated an *EDG-1* PCR amplification product of about 1300 base pairs, in agreement with the predicted size (1284 base pairs), in HUVEC and BAEC (Fig. 2, *B* and *C*). HUVEC and BAEC also apparently expressed somewhat lower levels of *EDG-3* and barely detectable *EDG-5* mRNA. It should be noted that the bovine SPP receptor cDNAs have not yet been cloned and sequenced; however, our PCR primers were designed to cover highly conserved regions. Restriction analysis of the HUVEC RT-PCR products yielded fragments of the expected sizes confirming their identity (data not shown). The entire open reading frame of each of the three SPP receptors is encoded within a single exon (40, 41), and RT-PCR primers which span an intron junction cannot be used to evaluate genomic DNA contamination. Therefore, controls without reverse transcriptase were performed in all cases (Fig. 2).

Effects of SPP on Chemotaxis of Endothelial Cells—Since SPP increased chemotaxis of HEK293 cells by apparently acting through EDG-1, the effect of SPP on chemotaxis of HUVEC and BAEC, which express EDG-1 and -3, was examined (Fig. 3, *A* and *B*). SPP stimulated chemotaxis of both HUVEC and BAEC, reaching a maximum effect at 1 μM (7- and 10-fold, respectively). Similar to the results with HEK293-*EDG-1* cells, concentrations of SPP greater than 1 μM were less effective, although significant enhancement of chemotaxis was still evident at higher concentrations. Interestingly, bFGF (20 ng/ml), a potent angiogenic factor (42), increased chemotaxis of BAEC to the same extent as did 1 μM SPP.

It has recently been shown that directional migration toward appropriate agonist ligands can be triggered via receptors coupled to G_i but not by agonists for receptors coupled to two other G proteins, G_s and G_q (43). Because biochemical evidence and the yeast two-hybrid system indicate that EDG-1 is capable of interaction with $G\alpha_{i1}$ and $G\alpha_{i3}$ (44), we investigated the possibility that G_i proteins may be involved in the chemotactic response induced by SPP. To this end, HUVEC were treated with pertussis toxin, which ADP-ribosylates and inactivates G_i and G_o proteins, prior to addition of SPP. Pertussis toxin pretreatment completely abolished SPP-induced chemotaxis (Fig. 3*C*).

SPP Stimulates Directional Migration—We next determined whether the effect of SPP was mediated by enhanced directed migration in response to the gradient of chemoattractant (chemotaxis) or by increased random motility due to the presence of the chemoattractant itself (chemokinesis). Checkerboard as-

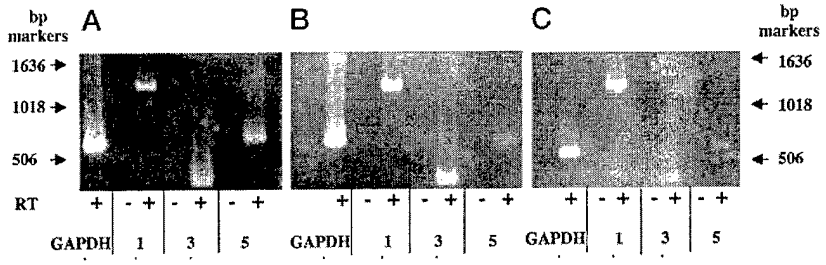

FIG. 2. **Expression of EDG receptor mRNAs in endothelial cells.** RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *EDG-1*, *EDG-3*, and *EDG-5* expression was carried out with RNA isolated from HEK293-*EDG-1* cells (*A*), HUVEC (*B*), or BAEC (*C*) as described under "Experimental Procedures" in the absence (−) or presence (+) of MULV-RT (RT). Similar results were found in two independent experiments. *bp*, base pair(s).

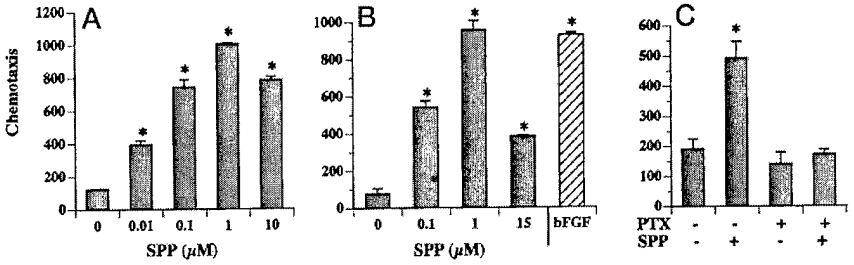

FIG. 3. **Effect of SPP on chemotaxis of HUVEC and BAEC.** *A*, HUVEC were allowed to migrate toward the indicated concentrations of SPP and chemotaxis was measured as described under "Experimental Procedures." *B*, BAEC were allowed to migrate toward different concentrations of SPP or 20 ng/ml bFGF as indicated, and chemotaxis was measured. Data are mean ± S.D. of triplicate determinations. Similar results were obtained in at least three independent experiments. *C*, HUVEC were pretreated with vehicle or 200 ng/ml pertussis toxin for 3 h, and then allowed to migrate toward vehicle or 100 nM SPP as indicated. *Asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$).

says were performed with various concentration of SPP in the top, bottom, or both chambers of the Boyden apparatus. The greatest numbers of cells were found to migrate either along the chemotactic gradient, *i.e.* toward increasing concentrations of SPP in the bottom chamber (Table I, *bold*), and also in the direction of the increasing chemokinetic gradient, *i.e.* when the concentration of SPP was the same in both the top and bottom chambers (Table I, *italic*), indicating that SPP stimulates both chemokinetic and chemotactic responses.

Binding of SPP and Dihydro-SPP Correlates with Stimulation of Cell Migration—It was important to determine whether [^{32}P]SPP was specifically bound to the endogenous SPP receptors on HUVEC. As shown in Fig. 4*A*, in HUVEC there was significant specific binding of both SPP and sphinganine 1-phosphate (dihydro-SPP), which lacks the double bond at the 4-position. Moreover, dihydro-SPP also markedly enhanced chemotaxis (Fig. 4*B*). The structure of SPP is similar to that of LPA, another serum-borne lysolipid that binds and signals through the related G_i-coupled receptors, EDG-2 and EDG-4 (45–47). However, excess LPA did not compete with [^{32}P]SPP for binding to HUVEC, nor did it have a significant stimulatory effect on chemotaxis. SPC had no significant effect on specific [^{32}P]SPP binding and had a small but not statistically significant effect on chemotaxis (Fig. 4, *A* and *B*). Similar results were obtained with BAEC, where only SPP and dihydro-SPP, albeit less potently, competed with labeled SPP for binding, whereas LPA and SPC had no significant effect. These results are consistent with our previous observation that dihydro-SPP blocked binding to HEK293 cells overexpressing EDG-1 in a dose-dependent manner similar to unlabeled SPP (9, 24), while neither LPA nor SPC had a significant effect on SPP binding (Fig. 1*B*). It should be pointed out that in these studies, all lysosphingolipids were added to cells as 0.4% BSA complexes, using conditions which were found to be optimal for binding of SPP to its receptor (9, 24), whereas these might not be optimal conditions for binding of LPA to EDG-2 and EDG-4. For example, LPA prepared as a 1% BSA complex promoted survival of Schwann cells, while SPP as a 0.01% complex was ineffective (48). We also examined the binding affinity of SPP for its putative receptor on endothelial cells by displacing bound [^{32}P]SPP with increasing concentrations of unlabeled SPP. 50% of the bound [^{32}P]SPP was competed at 10 nM unlabeled SPP in BAEC. Thus, binding of SPP to endothelial cells is also of high affinity and in agreement with the K_d of EDG-1 (8.6 nM). These results indicate that there is an excellent correlation between the K_d and the concentration-dependent effect of SPP on cell migration.

TABLE I
Checkerboard analyses of HUVEC migration induced by SPP

The checkerboard assay was arranged with increasing concentrations of SPP in the lower chamber (bold indicates migration due to chemoattaction) or increasing concentrations of SPP in both upper and lower chambers, and cell migration assays were performed as described under "Materials and Methods." Data are mean ± S.D. of triplicate determinations.

Lower chamber	Upper chamber			
	0	10	100	1000
nM				
0	117 ± 2	147 ± 24	177 ± 54	298 ± 14
10	376 ± 21	347 ± 24	317 ± 53	390 ± 32
100	708 ± 43	677 ± 18	616 ± 108	506 ± 7
1000	955 ± 7	1001 ± 17	904 ± 101	823 ± 24

SPP Stimulates Proliferation of HUVEC and BAEC—Many angiogenic factors, in addition to enhancing chemotaxis, stimulate *in vitro* proliferation of endothelial cells (49–51). Since SPP increased chemotaxis of endothelial cells, and has previously been shown to be a potent mitogen for diverse cell types (1, 2, 52), it was of interest to examine the effects of SPP on proliferation of endothelial cells. SPP treatment of HUVEC induced a dose-dependent increase of DNA synthesis as measured by [^{3}H]thymidine incorporation with a maximum effect at 0.1–1 μM (Fig. 5*A*). Similar to SPP, 1 μM dihydro-SPP also stimulated DNA synthesis in HUVEC by 1.83 ± 0.1-fold, whereas LPA at a concentration as high as 10 μM had no

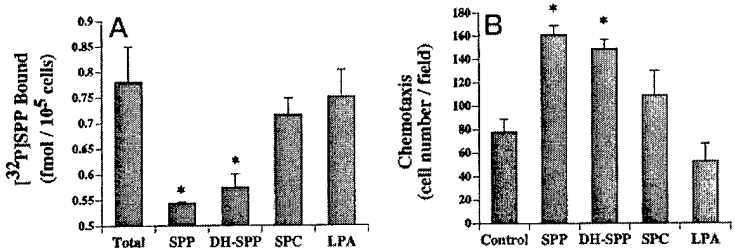

FIG. 4. **SPP and dihydro-SPP, but not LPA, specifically compete with [^{32}P]SPP binding and stimulate chemotaxis in HUVEC.** *A*, effects of SPP and other lysophospholipids on binding of [^{32}P]SPP to HUVEC. HUVEC were incubated in the presence of 0.2 nM [^{32}P]SPP for 30 min at 4 °C in the absence or presence of 300 nM unlabeled SPP, dihydro-SPP (DH-SPP), SPC, or LPA and binding was measured as described under "Experimental Procedures." *B*, HUVEC were allowed to migrate toward the indicated lysophospholipids (100 nM) and chemotaxis was measured. *Asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$).

significant effect. In agreement with our previous reports in various cell types (53–55), 10 μM SPC stimulated DNA synthesis by 1.95 ± 0.1-fold.

SPP was also mitogenic for BAEC; however, a maximal effect in these cells required higher concentrations (1–10 μM). bFGF has been reported to be a potent endothelial cell mitogen (56). Surprisingly, although bFGF stimulated proliferation of BAEC, at optimal concentrations it was only as effective as 1 μM SPP and less effective than 10 μM SPP (Fig. 5*B*). It should be noted that sensitivity of BAEC to SPP decreased with increasing passage number, similar to a previous report on the effect of passage number on bFGF responses (57). In addition to SPP, dihydro-SPP stimulated DNA synthesis, whereas we found that LPA at a concentration up to 10 μM was not mitogenic, in fair agreement with previous studies where LPA only stimulated DNA synthesis in BAEC at concentrations around 30 μM (57).

To investigate the possibility that a G_i-coupled receptor may be involved in the proliferative response induced by SPP, endothelial cells were treated with pertussis toxin prior to addition of SPP. Both HUVEC and BAEC are more sensitive to pertussis toxin than Swiss 3T3 fibroblasts. In contrast with our previous studies with quiescent Swiss 3T3 fibroblasts (10, 11), where half of the stimulated DNA synthesis was still evident even at the highest effective concentration of pertussis toxin, pertussis toxin pretreatment of HUVEC and BAEC completely inhibited the SPP-induced mitogenic response, while it had no significant effect on DNA synthesis induced by bFGF (Fig. 5*C*).

SPP Induces Capillary-like Tube Formation in Vitro—Later stages of angiogenesis require morphological alterations of endothelial cells, which result in lumen formation (31). Critical steps in angiogenesis, such as migration and differentiation, have been studied using an *in vitro* model of angiogenesis in which cultured endothelial cells are induced to invade a three-dimensional collagen gel where they form a network of capillary-like structures or tubes when stimulated by angiogenic factors (58, 59). This phenomenon is thought to mimic the formation of new blood vessels *in vivo*. In agreement with previous studies (59), confluent monolayers of BAEC treated with bFGF (50 ng/ml) formed networks of capillary-like tubular structures within the gel (Fig. 6*A*). In contrast, little invasion or network cord formation and only a few short capillary sprouts originating from untreated BAEC embedded in collagen gels were detected. SPP evoked a dose-dependent increase in the formation of capillary-like tubes of BAEC invading the collagen gel. Apparently thinner tubes were formed in response to lower doses of SPP than those formed in response to bFGF. Quantitative evaluation of tube formation revealed that SPP, similar to bFGF, markedly increased the length of the endothelial tubular structures (Fig. 6*B*). There was an additive effect when SPP was applied together with bFGF, suggesting

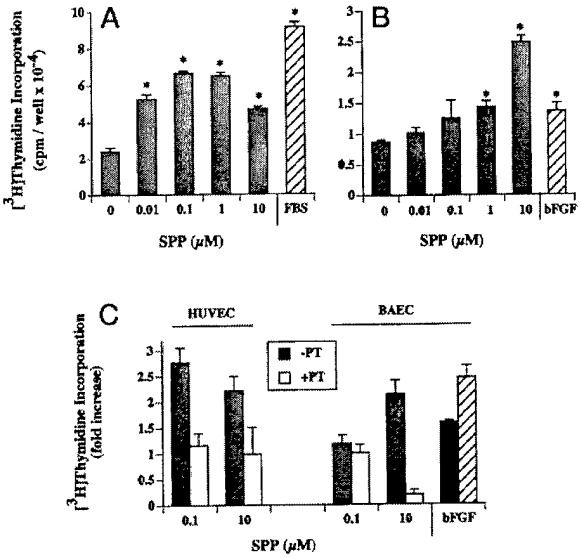

FIG. 5. **The effect of SPP on DNA synthesis of HUVEC and BAEC.** Quiescent HUVEC (*A*) or BAEC (*B*) were treated with the indicated concentrations of SPP or 20 ng/ml bFGF for 24 h and DNA synthesis as measured by [^{3}H]thymidine incorporation was determined as described under "Experimental Procedures." Data are mean ± S.D. of triplicate determinations and are representative of at least three independent experiments. *Asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$). *C*, quiescent HUVEC and BAEC were incubated in the absence (*filled bars*) or presence of 20 ng/ml pertussis toxin (*open* or *hatched bars*). After 2 h, cells were washed and exposed to the indicated concentrations of SPP or bFGF for 24 h and [^{3}H]thymidine incorporation was measured. Data are expressed as fold increases compared with non-stimulated cells.

that SPP can potentiate the effect of bFGF on *in vitro* angiogenesis. Similar to SPP, dihydro-SPP, also markedly enhanced capillary-like tube formation of BAEC, whereas LPA was completely inactive and SPC had a small but significant effect (Fig. 6*C*). Pertussis toxin not only inhibited endothelial cell migration and proliferation induced by SPP, it also markedly decreased the SPP-induced tube formation (Fig. 6*C*). In sharp contrast, pertussis toxin had no effect on tube formation induced by bFGF.

DISCUSSION

Previously, many studies have shown that SPP inhibits chemotaxis of diverse cell types (17, 19, 20, 60, 61). Although in human breast cancer cells, SPP inhibited chemotaxis independently of EDG-1 (21), a wealth of evidence suggests that in many other cell lines, SPP inhibits chemotaxis through unidentified cell surface receptors (17, 20, 62). Unexpectedly, we found

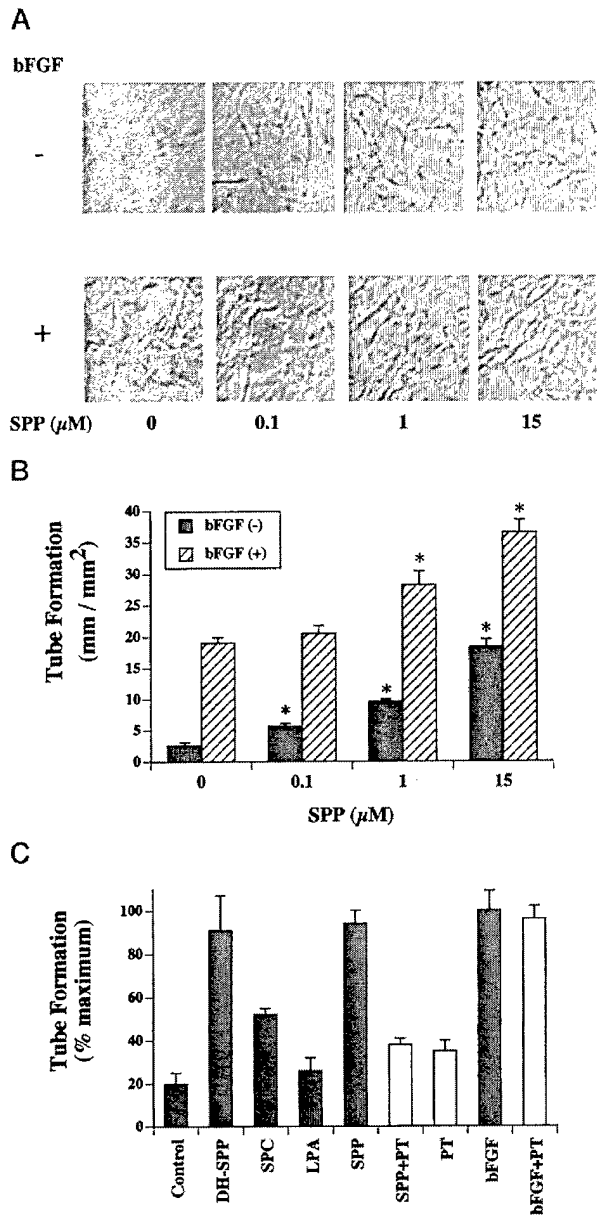

FIG. 6. **Induction of *in vitro* angiogenesis in collagen gels by SPP is attenuated by pertussis toxin.** BAEC were treated with the indicated concentration of SPP in the absence or presence of bFGF (50 ng/ml) and capillary tube formation on collagen gels was examined as described under "Experimental Procedures." *A*, representative phase-contrast micrographs of BAEC after 4 days of incubation in normal medium (*upper panels*) or in the presence of 50 ng/ml bFGF (*lower panels*) and the indicated concentrations of SPP. *B*, quantitative analysis of capillary tube formation. Data are expressed as length of tubes per square millimeter (n = 4 pairs of duplicates). *Asterisks* indicate statistical significance determined by Student's t test ($p \leq 0.01$). *C*, BAEC were treated in the absence or presence of pertussis toxin (*PT*, 20 ng/ml), without or with the indicated lysophospholipids (1 μM) or bFGF (50 ng/ml) and capillary tube formation on collagen gels was determined as described under "Experimental Procedures." Data are expressed relative to the maximum response elicited by bFGF.

in this study that binding of SPP to its G_i protein-coupled receptor EDG-1 markedly increased cell motility. In accord with its affinity for EDG-1, SPP at nanomolar concentrations increased chemotaxis of EDG-1-transfected but not vector-transfected HEK293 cells, as well as HUVEC and BAEC which constitutively express EDG-1.

Although a recent study demonstrated that LPA can bind to EDG-1 leading to receptor phosphorylation, ERK activation, as well as Rho-dependent morphogenesis and P-cadherin expression (39), in our study, LPA, even at a concentration as high as 10 μM, did not compete for binding of radiolabeled SPP to HEK293-*EDG-1* cells. In agreement, LPA did not displace bound [^{32}P]SPP from endothelial cells nor did it stimulate chemotaxis of HUVEC. In contrast, dihydro-SPP, which binds to EDG-1 (9), EDG-3, and EDG-5 (24), was as potent as SPP in induction of chemotaxis. However, LPA, similarly to SPP, can mimic serum in inducing invasion of carcinoma and hepatoma cells into monolayers of mesothelial cells (63). Although the underlying mechanism of this effect is not clear, it may involve increased cell adhesion rather than enhanced cell motility. Moreover, in most other cell types, LPA stimulates chemokinesis and chemotaxis (62, 64–66), which might be due to binding to its specific receptors, EDG-2 and EDG-4 (45–48).

We have previously shown that the SPP-induced cAMP decrease (9) and ERK2 activation in EDG-1-transfected cells (22) were completely blocked by pretreatment with pertussis toxin, which uncouples G_i from GPCR. Similarly, preincubation with pertussis toxin abolished the effect of SPP on migration of endothelial cells. Collectively, these findings suggest that binding of SPP to the serpentine receptor EDG-1 on the endothelial cell surface activates a pertussis toxin-sensitive G_i protein crucial for chemotaxis. In agreement, it has recently been demonstrated that activation of $G\alpha_i$-coupled receptors and the subsequent release of $G_{\beta\gamma}$ dimers is required to initiate signal transduction leading to directed cell migration (43, 67). It should be noted that the receptors for all known leukocyte chemoattractants, including the chemokines, are members of a seven-transmembrane domain superfamily and coupled to a variety of G_α subunits (68, 69). Moreover, pertussis toxin inhibits chemotaxis by preventing chemoattractant receptors from activating trimeric G proteins of the G_i subfamily (67).

Endothelial cells are accessible to platelet-derived ligands in the serum, and might be the target of serum-borne SPP which regulates their proliferation, migration, and differentiation into capillary-like tubules, important aspects of angiogenesis (31). SPP is well established as a potent mitogen for diverse cell types (reviewed in Ref. 1). Indeed, we found that SPP also stimulated DNA synthesis in endothelial cells and this was completely blocked by pertussis toxin, suggesting a role for G_i in this process. In agreement, SPP was recently reported to stimulate HUVEC proliferation (70), albeit at somewhat higher concentrations which resembled the dose-response curve that we found for BAEC. It is possible that differences in sensitivity to SPP might arise from differences in passage numbers as has been shown for responses to bFGF (57). Interestingly, SPP stimulated DNA synthesis in Swiss 3T3 fibroblasts only at high concentrations and in contrast to endothelial cells, was only partially inhibited by pertussis toxin (10). Moreover, in these cells, DNA synthesis was significantly and specifically increased by microinjection of SPP (9). Pertussis toxin reduced the level of DNA synthesis caused by exogenous SPP to approximately the same level as that induced by microinjected SPP (9). Thus it is likely that in fibroblasts, both intracellular as well as receptor-mediated responses to SPP are involved in its mitogenic effect. It is possible that there is a complex interplay between cell surface receptor signaling and intracellular targets for SPP, which can contribute to its mitogenic response in certain cell types. Thus, SPP, may act in a similar manner to other bioactive lipids, such as leukotriene B4 (LTB4), which act through cell surface receptors and also have intracellular targets. LTB4 is a potent chemoattractant that is primarily in-

volved in activation of inflammatory cells by binding to its GPCR. However, it can also bind and activate the intranuclear transcription factor PPARα, resulting in the activation of genes that terminate inflammatory processes (71).

In this study, we also observed that SPP in addition to stimulating both random, nondirectional migration (chemokinesis) and directional migration (chemotaxis) of endothelial cells, also markedly enhanced morphogenetic differentiation of endothelial cells *in vitro*. BAEC cultured on collagen showed increased tube formation in the presence of SPP, demonstrating that SPP stimulates endothelial cell differentiation as well as migration. Moreover, pertussis toxin not only inhibited endothelial cell migration and proliferation induced by SPP, it also completely inhibited SPP-induced, but not bFGF-induced, formation of capillary-like tubes. Taken together, these results demonstrate that SPP has angiogenic activity *in vitro* acting through a G_i-coupled cell surface receptor.

In contrast to its effect on HEK293-*EDG*-1 cells, SPP did not stimulate chemotaxis (Fig. 1), proliferation (9), or morphogenetic differentiation in vector-transfected HEK293 cells (22), further supporting the notion that the effects that we observed on chemotaxis, proliferation, and tube formation of endothelial cells are mediated by EDG-1. In contrast, parental and vector-transfected HEK293 cells do show activation of ERK mitogen-activated protein kinases in response to SPP which might be attributable to endogenous EDG-3 or EDG-5. Taken together with our results, these findings suggest that EDG-1 may mediate these stimulatory effects of SPP on endothelial cells. However, as the other known SPP receptor EDG-3, which is also expressed in HUVEC, has been shown in some cases to couple to pertussis toxin-sensitive G proteins as well as to Rho and phospholipase C signaling pathways (14, 26), this receptor might also contribute to the effects of SPP on tube formation. Recently, the polycyclic anionic compound suramin has been shown to selectively antagonize SPP-activated calcium transients in EDG-3, but not in EDG-1 or EDG-5 expressing oocytes, with an IC_{50} of ~22 μM, suggesting that it is an antagonist selective for the EDG-3 GPCR isotype (72). However, addition of 100 μM suramin did not abrogate the ability of SPP to induce tube formation in BAEC.[2] In contrast, suramin inhibited Rho-dependent neurite retraction induced by SPP in N1E-115 neuronal cells (73) and SPP-induced invasion of T-lymphoma cells (74). However, in agreement with its lack of effect on SPP-induced tube formation, suramin had no significant effect on proliferation, stress fiber formation, and focal adhesion kinase phosphorylation induced by SPP in Swiss 3T3 fibroblasts (75).

Similar to SPP, dihydro-SPP also markedly enhanced cell migration and capillary-like tube formation of BAEC, whereas LPA was completely inactive. Another structurally related analog of SPP, SPC, although it had no significant effect on binding of labeled SPP to endothelial cells, it had a small but significant stimulatory effect on tube formation, and at high concentrations, it also stimulated proliferation. These effects might be related to the potent action of SPC as a wound healing agent (55). Interestingly, high micromolar concentrations of SPC activated calcium transients in EDG-1, -3, and -5 expressing oocytes (72) and SRE-driven gene transcription in Jurkat T cells (25). While these observations suggest that SPC might be a very low affinity ligand for these EDG receptors, it is also possible that these effects were not mediated by SPC itself as it was recently found that commercial preparations of SPC are contaminated with highly potent alkenyl glycero-3-phosphates (76).

[2] J. P. Hobson and S. Spiegel, unpublished observation.

Some of the downstream effects of SPP signaling through EDG-1, such as decreased cAMP (9, 23) and activation of ERK2 (22), are pertussis toxin-sensitive, while others, such as morphogenetic differentiation, are pertussis toxin-insensitive but inhibited by the C3 exoenzyme (22) which blocks signaling through the small GTPase Rho (77). Thus it appears that EDG-1 can couple to G_i proteins as previously reported (44), as well as to $G_{12/13}$ proteins which are thought to regulate Rho (78, 79), and thus might also be important for SPP-induced chemotaxis and angiogenesis. Recently, it has been demonstrated that disruption of the gene encoding $G\alpha_{13}$ impaired the ability of endothelial cells to organize into a vascular system and greatly impaired migratory responses (80), suggesting that in addition to G_i, other proteins including $G\alpha_{13}$, might be required for regulation of cell movement. In agreement, T-lymphoma cell invasion is dependent on SPP receptor-mediated RhoA and phospholipase C signaling pathways which lead to pseudopodia formation (74).

In summary, in this study we have demonstrated that SPP has appropriate properties to be considered as a *bona fide* angiogenic factor, *i.e.* it stimulates chemokinetic and chemotactic motility, proliferation of vascular endothelial cells, and stimulates angiogenesis *in vitro*, similarly to the known angiogenic factor bFGF. Because bFGF and SPP have an additive effect on formation of capillary-like tubes by endothelial cells invading collagen gels, SPP may be a specific type of angiogenic factor. It is possible that SPP plays a role in normal blood vessel formation or in injury, when local production of SPP could be increased by activated platelets, and extravasation of intravascular fluid could also present SPP into tissues at concentrations sufficient to promote angiogenesis and wound healing. Elucidation of the molecular mechanisms by which SPP stimulates cell migration and angiogenesis might provide clues for development of new therapeutic agents to either promote or block these processes by targeting the EDG family of GPCR.

Acknowledgments—We thank Dr. Luyuan Li and the Lombardi Cancer Center Microscopy/Imaging shared resources, supported by U. S. Public Health Service Grant 1P30-CA-51008, for providing use of microscopy facilities. We also thank Drs. Timothy Hla and Menq-Jer Lee for providing HEK293-*EDG-1* cells.

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Edg-1, the G protein–coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation

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Received for publication July 28, 2000, and accepted in revised form September 12, 2000.

Sphingolipid signaling pathways have been implicated in many critical cellular events. Sphingosine-1-phosphate (SPP), a sphingolipid metabolite found in high concentrations in platelets and blood, stimulates members of the endothelial differentiation gene (Edg) family of G protein–coupled receptors and triggers diverse effects, including cell growth, survival, migration, and morphogenesis. To determine the in vivo functions of the SPP/Edg signaling pathway, we disrupted the *Edg1* gene in mice. *Edg1*$^{-/-}$ mice exhibited embryonic hemorrhage leading to intrauterine death between E12.5 and E14.5. Vasculogenesis and angiogenesis appeared normal in the mutant embryos. However, vascular maturation was incomplete due to a deficiency of vascular smooth muscle cells/pericytes. We also show that Edg-1 mediates an SPP-induced migration response that is defective in mutant cells due to an inability to activate the small GTPase, Rac. Our data reveal Edg-1 to be the first G protein–coupled receptor required for blood vessel formation and show that sphingolipid signaling is essential during mammalian development.

J. Clin. Invest. **106**:951–961 (2000).

Introduction

Sphingolipids have emerged as important signaling molecules in a variety of biologic processes (1–3). SPP in particular has come to the fore as a mediator of an extracellular signaling pathway through its interaction with the family of G protein–coupled receptors known by the acronym, Edg (endothelial differentiation gene) (4). Edg-1, the first of these receptors described, was identified as a gene induced during human endothelial cell differentiation (5). Activation of the Edg receptors triggers diverse effects including proliferation, survival, migration, morphogenesis, adhesion molecule expression, and cytoskeletal changes and has led to the view that the Edg receptor signaling pathways may have important roles in many physiological and pathological events (reviewed in refs. 6–10).

The Edg family can be subdivided into either receptors for SPP or for lysophosphatidic acid. The Edg receptors for SPP activate different and sometimes overlapping G protein–mediated intracellular signaling pathways. For instance, Edg-1 couples directly to the G_i pathway, whereas Edg-3 and -5 stimulate G_i, G_q, and G_{13} pathways with differing degrees of potency (4, 11–14). Moreover, the expression pattern of individual Edg receptors changes during development and differentiation, leading to different combinations on cells and tissues (15–18). The diverse receptor expression and activation of divergent signaling pathways may explain the pleiotropic responses to SPP but have made functional analysis difficult.

To determine the functions of the SPP/Edg-1 signaling pathway, we have disrupted *Edg1* in mice. Homozygous *Edg1* mutant mice die in utero due to massive embryonic hemorrhage. They undergo normal vasculogenesis and angiogenesis but are severely impaired in vessel maturation due to a defect in the recruitment of mural cells to vessel walls. The results reveal the SPP receptor Edg-1 as mediating a novel G protein–coupled signaling pathway required for blood vessel development.

Methods

Generation of Edg1 mutant mice. To generate the *Edg1* knockout mice, we cloned a 10-kb genomic DNA fragment containing the entire *Edg1* gene from a 129/Sv

library. As shown in Figure 1a, the *Edg1* gene is composed of two exons and an intron (16). The second large exon contains a 5′-UTR region, the entire open reading frame region, and approximately 1.8 kb of the 3′-UTR region. For knocking-in the *LacZ* reporter gene and targeted inactivation of the *Edg1* gene, a NcoI site in the beginning of the *Edg1* open reading frame was used to insert a *LacZ-neo* (neomycin-resistant gene) cassette (19). In the construct used for disruption of the *Edg1* gene, the *LacZ* coding region is preceded by an internal ribosomal entry sequence (20). Therefore, targeted insertion generates a bi-cistronic transcription unit in which the expression of the β-galactosidase reporter protein is under the control of *Edg1* transcriptional regulatory elements. The herpes simplex virus thymidine kinase (TK) gene was located outside the homologous sequence to prevent random integration.

Gene targeting in TC1 embryonic stem (ES) cells and generation of chimeric and heterozygous mice were as described previously (21). One targeted ES clone was used to establish chimeric mice, which were crossed with C57BL/6 mice to obtain *Edg1* heterozygotes. All mice analyzed were obtained from intercrosses of the *Edg1* heterozygotes. *Edg1* genotypes were determined by Southern blot and PCR analyses of genomic DNA isolated from ES cells, yolk sacs and tail biopsies. For genotyping by PCR, the primers were: 5′TAGCAGCTATGGTGTCCACTAG3′ (Primer 1), 5′GATCCTGCAGTAGAGGATGGC3′ (Primer 2), 5′TTGGAGTGACGGCAGTTATCTGGA3′ (Primer 3), and 5′TCAACCACCGCACGATAGAGATTC3′ (Primer 4). Primers 1 and 2 detected the wild-type *Edg1* allele and amplified an approximately 630-bp fragment. Primers 3 and 4 detected the $Edg1^{LacZ}$ allele and amplified an approximately 350-bp fragment. Forty-five cycles of 94°C (1 minute), 55°C (1 minute), and 72°C (3 minutes) were used.

Histological analysis. Embryos at embryonic days (E) 9.5–16.5 were removed from the mother after heterozygous mating. Then the embryos were fixed and processed to be embedded in paraffin. Serial sections (5-μm-thick) were made at 15-μm intervals and stained with hematoxylin and eosin (H&E).

Paraffin sections were deparaffinized and rehydrated. Antigen retrieval was accomplished by 30-minute incubation at 95°C in Target Retrieval Solution (DAKO Corp., Carpenteria, California, USA). Endogenous peroxidase activity was quenched by incubation with 5% hydrogen peroxide in methanol for 5 minutes. Specimens were incubated with anti-smooth muscle α actin (EPOS anti-SMαA/HRP; no. U7033; DAKO Corp.) for 1 hour at room temperature. After washing with PBS, peroxidase reaction was visualized with diaminobenzidine/hydrogen (DAB/hydrogen) peroxide.

To define the developmental and tissue-specific expression patterns of *Edg1* through X-Gal staining, embryos dissected out from the decidua at various developmental stages were fixed in 2% formaldehyde/2% glutaraldehyde in PBS for 10 minutes. They were washed in PBS and then incubated in PBS containing 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-Gal at 37°C overnight. Reactions were stopped by rinsing embryos with PBS, followed by further fixation in 4% paraformaldehyde.

Whole-mount embryo immunostaining. Embryos were dissected out and fixed in 4% paraformaldehyde in PBS at 4°C overnight. They were then dehydrated through a methanol series and stored in 100% methanol at –20°C. The embryos were bleached in 6% hydrogen peroxide/methanol for 1 hour at room temperature and rehydrated through a methanol series to PBS + 0.1% Tween 20 (PBST). They were incubated in a blocking solution (4% BSA in PBST) twice, for 1 hour each time. The embryos were incubated with rat mAb's (anti–PECAM-1: no.1951D; anti-CD34: no. 09431D; anti–VE-cadherin: no. 28091D; PharMingen, San Diego, California, USA), diluted 1:200 in 10% goat serum and 4% BSA in PBST at 4°C overnight. Embryos were washed with 4% BSA in PBST at room temperature and then incubated with peroxidase-conjugated goat anti-rat Ig in 10% goat serum and 4% BSA in PBST at 4°C overnight. Peroxidase reaction was visualized with DAB/hydrogen peroxide.

RT-PCR and immunoblotting. Total RNA was isolated from E12.5 mouse embryos and cultured cells using Trizol (Life Technologies Inc., Gaithersburg, Maryland, USA) and treated with DNaseI (Life Technologies). Total RNA (5 μg) was reverse transcribed using Superscript Preamplification System (Life Technologies) according to the manufacturer's instructions. PCR was performed on 2 μl of the RT reaction in a volume of 50 μl using AmpliTaq Gold polymerase (Perkin-Elmer Corp., Norwalk, Connecticut, USA). The PCR conditions were as follows: initial denaturation at 95°C for 10 minutes followed by up to 35 cycles of denaturation at 95°C (1 minute), annealing at 55°C (1 minute), and extension at 72°C (1 min). Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. PCR primer pairs were as follows:

Flt-1: (5′TGTGGAGAAACTTGGTGACCT3′, 5′TGGAGAACAGCAGGACTCCTT3′)
Flk-1: (5′TCTGTGGTTCTGCGTGGAGA3′; 5′GTATCATTTCCAACCACCCT3′)
Tie-1: (5′TCTTTGCTGCTCCCCACTCT3′, 5′ACACACACATTCGCCATCAT3′)
Tie-2: (5′CCTTCCTACCTGCTACTTTA3′, 5′CCACTACACCTTTCTTTACA3′)
Ang-1: (5′AAGGGAGGAAAAAGAGAAGAAGAG3′, 5′GTTAGCATGAGAGCGCATTTG3)
Ang-2: (5′TGCCTACACTACCAGAAGAAC 3′, 5′TATTTACTGCTGAACTCCCAC 3′)
PECAM-1: (5′GTCATGGCCATGGTCGAGTA3′, 5′CTCCTCGGCATCTTGCTGAA3′)
VE-cadherin: (5′GGATGCAGAGGCTCACAGAG3′, 5′CTGGCGGTTCACGTTGGACT3′)
Smad5: (5′CTTTCCACCAACCCAACAAC3′, 5′TCATAGGCGACAGGCTGAAC3′)
endoglin: (5′TACTCATGTCCCTGATCCAGCC3′,

5′GTCGATGCACTGTACCTTTTTCC3′)
LKLF: (5′CCACACATACTTGCAGCTACAC3′,
5′CCATCGTCTCCCTTATAGAAATA3′)
Edg-1: (5′TAGCAGCTATGGTGTCCACTAG-3′,
5′GATCCTGCAGTAGAGGATGGC3′)

For immunoblotting, detergent extracts of E12.5 embryos were analyzed for protein expression using antibodies against the following proteins: VE-cadherin (catalog no. 28091D; PharMingen); N-cadherin (catalog no. SC-7939; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA); and P-cadherin (catalog no. SC-7893), PDGF-B (catalog no. SC-7878), and VEGF (catalog no. SC-507; all from Santa Cruz Biotechnology). A peroxidase-conjugated secondary antibody was used, and the reaction was visualized with the ECL + Plus Western blotting system (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

In vitro assays. Chemotactic migration of cells was measured in a modified Boyden chamber as described previously using polycarbonate filters (25 × 80 mm; 12 μm pore size) coated with collagen type I (50 μg/ml in 5% acetic acid), which promotes uniform attachment to and migration across the filter without formation of a barrier (22). SPP or medium without serum was placed in the lower chamber as chemoattractants. Mouse embryonic fibroblasts were harvested and added to the upper chamber at 5×10^4 cells per well. Each data point was the average number of cells in four random fields, each counted twice. Each determination represents the average ± SD of three individual wells. For the detection of GTP-bound activated Rac, embryonic fibroblasts were serum-starved for 24 hours before they were treated with SPP (200 nM) for 5 minutes. The cell lysates were used for affinity precipitation with the PAK-1 p21 binding domain-conjugated (PDB-conjugated) agarose beads (Upstate Biotechnology Inc., Lake Placid, New York, USA) as described elsewhere (23), or were used without fractionation to determine total Rac levels. Rac was visualized by immunoblotting with an mAb.

Results

Generation of Edg1 knockout mice with an inserted β-galactosidase (LacZ) reporter gene. Edg1 consists of two exons (16), with the entire coding region in the second exon (Figure 1a). To disrupt *Edg1* in mouse ES cells, we constructed a replacement-type targeting vector in which the *Edg1* coding region, containing 382 amino acids, was disrupted after the first 42 amino acids by a *LacZ-Neo* cassette containing a internal ribosome entry sequence (19). This targeting strategy should both create a disrupted *Edg1* allele and enable analysis of *Edg1* expression in mice by creation of an *Edg1-LacZ* hybrid transcript driven by the endogenous Edg-1 promoter elements. The targeting vector was linearized and electroporated into the TC-1 ES cell line. Genomic DNA from G418- and ganciclovir-resistant clones was analyzed by Southern blotting. Of the 120 clones examined, about 60% contained the 2.5-kb BamHI band diagnostic of a homologous recombination event. Targeted ES cells were injected into C57BL/6 mouse blastocysts and produced highly chimeric male mice that transmitted the targeted allele through the germ line (Figure 1b). No $Edg1^{LacZ}$ homozygotes were found among more than 100 newborn animals from heterozygous crosses (Table 1), indicating an embryonic lethal phenotype. Heterozygous *Edg1* mice appeared at the expected frequency and were phenotypically normal.

$Edg1^{LacZ}$ homozygous embryos were present in utero as determined by Southern analysis. To confirm that *Edg1* was disrupted by the targeting scenario, RT-PCR was performed on total embryonic RNA using primers flanking the disrupted sequence (RT-5′ and RT-3′ in Figure 1a). The predicted 630-bp product was amplified from RNA of both wild-type and heterozygous embryos (Figure 1c). No PCR amplified band was detected from RNA of homozygous mutant embryos, indicating disruption of the normal *Edg1* transcripts.

Expression patterns of Edg1 gene during mice embryogenesis. LacZ expression, under the control of native *Edg1* regulatory elements, was visualized by X-Gal staining in heterozygous embryos. At E9.5, intense expression was observed in the common ventricular chamber of heart, dorsal aorta, intersomitic arteries, and capillaries (Figure 1d). Weak expression was detected in the forebrain and common atrial chamber of the heart. Very weak or no expression was found in the anterior and posterior cardinal veins (Figure 1d). In addition to the expression pattern observed in the E9.5 embryo, E10.5 embryos showed prominent LacZ expression in the forebrain and a weaker level of expression in the spinal cord (Figure 1e). To examine the expression of the *Edg1* gene in detail, histological sections of X-Gal stained embryos were evaluated. The expression was most prominent in the endothelial cells of arteries and capillaries (Figure 1f), cardiomyocytes, and neuronal cells of the telencephalon (data not shown). No expression was found in the endothelial cells of veins (Figure 1g). Low levels of expression were detected the smooth muscle layers surrounding the aorta (Figure 1h).

Characterization of $Edg1^{-/-}$ embryos. To determine the time of embryonic lethality, embryos at various stages of gestation were isolated. Genotyping of E9.5 to E11.5 embryos from *Edg1* heterozygous intercrosses

Table 1
Genotype analysis of offspring from *Edg1* heterozygous intercrosses

Age	Total	Genotype +/+	Genotype +/−	Genotype −/−	(%)
E9.5	74	19	31	24	(32)
E10.5	42	8	23	11	(26)
E11.5	30	8	14	8	(27)
E12.5	113	32	55	26[A]	(23)
E13.5	45	9	28	8[A]	(18)
E14.5	33	12	21	0	(0)
E15.5-16.5	35	12	23	0	(0)
Adult	115	38	77	0	(0)

[A]Hemorrhage.

revealed inheritance of the *Edg1* mutant allele at the expected mendelian frequency (Table 1). Up to E11.5, *Edg1*$^{-/-}$ embryos appeared phenotypically normal. At E12.5, the *Edg1*$^{-/-}$ embryos could be identified by their abnormal yolk sacs, which were edematous, with less blood in the otherwise normal looking, highly branched, vasculature (Figure 2a, arrows). After removing the yolk sac, intraembryonic bleeding was evident in the *Edg1*$^{-/-}$ embryos (Figure 2b). The pericardial cavity of mutant embryos was enlarged and filled with fluid. The limbs of mutant embryos were underdeveloped and rounded with areas of bleeding. In comparison, age-matched wild-type embryos had more developed, fan-shaped limbs. Much less blood was found in the yolk sac blood vessels of E13.5 *Edg1*$^{-/-}$ embryos compared with those of the mutant embryos obtained 1 day earlier (Figure 2c, arrows). At E13.5, massive intraembryonic bleeding could be observed through the yolk sac. In addition to widespread hemorrhage, severe edema was observed

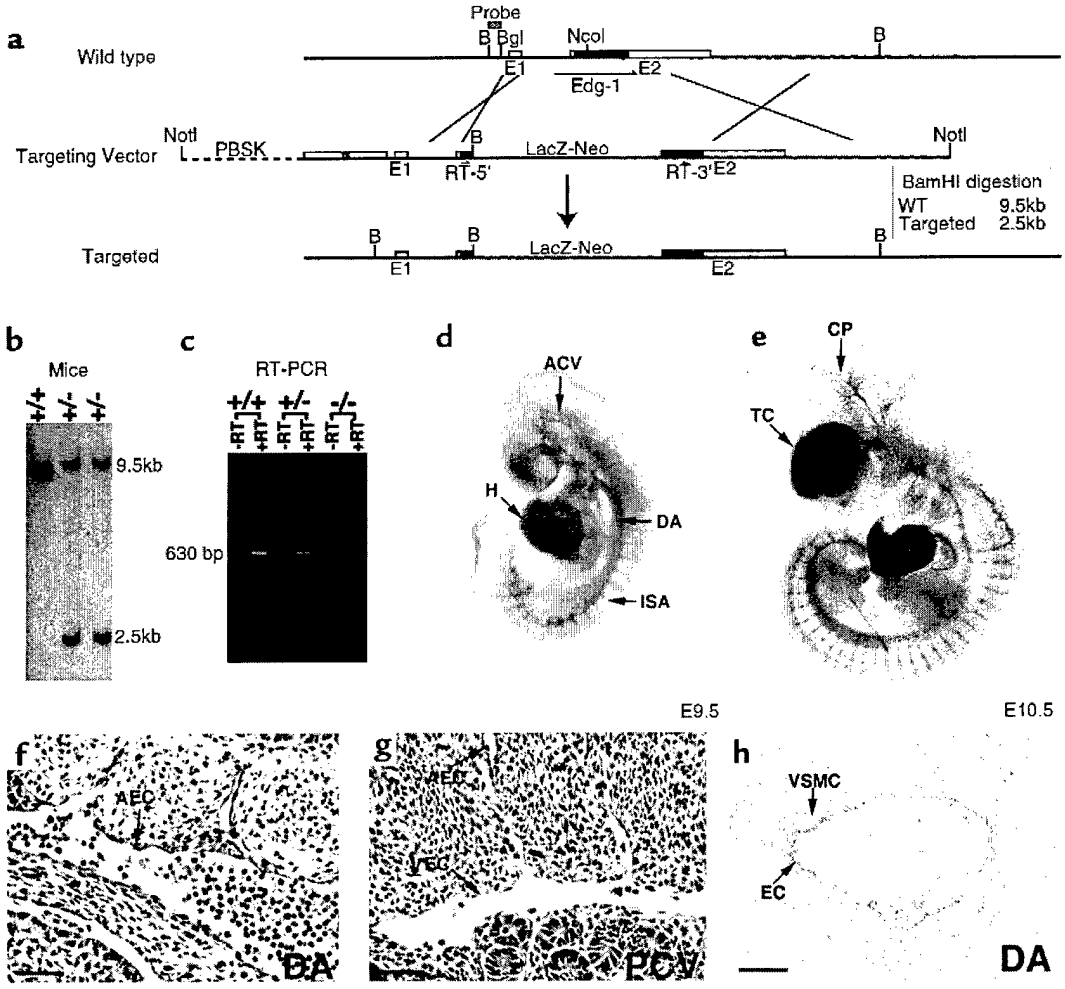
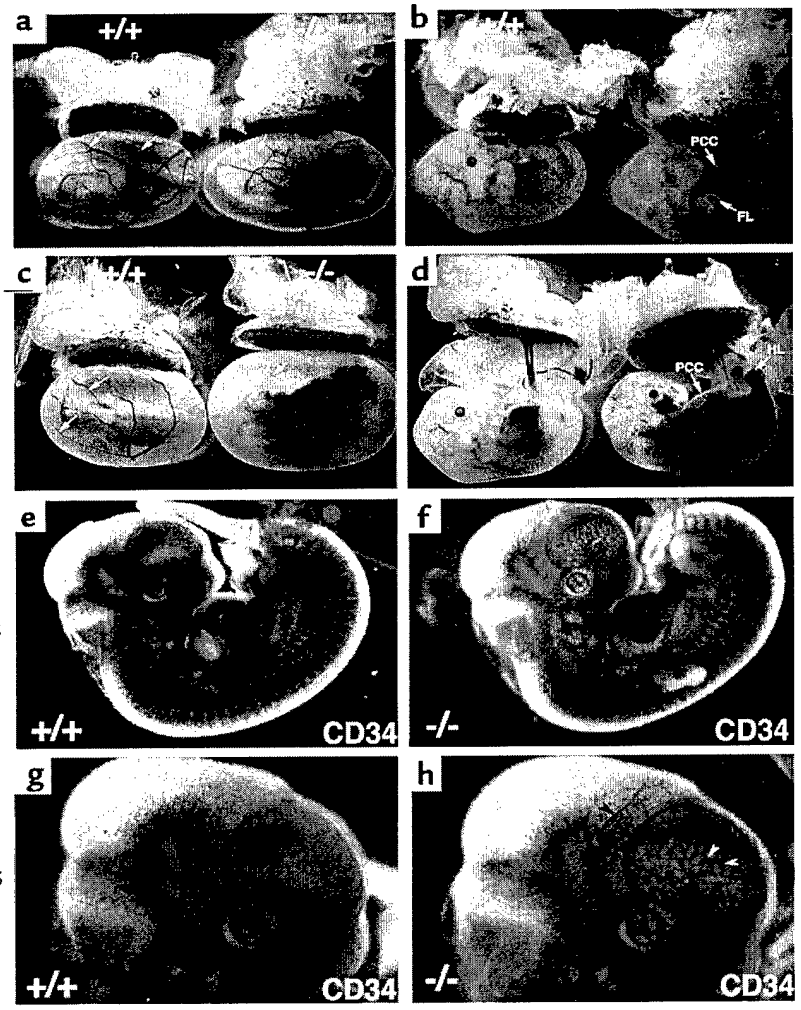


Figure 1

Targeted disruption and embryonic expression of the *Edg1* gene. (**a**) Schematic representation of the *Edg1* targeting strategy. The structure of the mouse *Edg1* locus is shown at the top, the structure of the *Edg1* targeting vector in the middle, and the predicted structure of the homologous recombined locus on the bottom. RT-5′ and RT-3′, primers for RT-PCR. B, BamHI; BglI, BglII; PBSK, pBluescript vector. (**b**) Genotyping of mouse offspring from the *Edg1* heterozygous mating. Wild-type *Edg1* locus yielded a 9.5-kb BamHI band. Disrupted *Edg1* locus yielded a 2.5-kb BamHI band. No *Edg1*$^{-/-}$ mice were found born alive. (**c**) RT-PCR analysis of total RNA from E12.5 mouse embryos by using RT-5′ and RT-3′. *Edg1*$^{+/+}$ and *Edg1*$^{+/-}$ RNA yielded the predicted 630-bp amplification product. No amplification product was detected from *Edg1*$^{-/-}$ RNA. (**d**, **e**) Whole-mount of *Edg1*$^{+/-}$ E9.5 and E10.5 embryos stained with X-Gal. H, heart; DA, dorsal aorta; ISA, intersomatic arteries; CP, capillaries; TC, telencephalon; ACV, anterior cardinal vein. (**f**) Longitudinal section of dorsal aorta (DA) from E10.5 *Edg1*$^{+/-}$ embryo. LacZ staining is seen in arterial ECs (AEC). (**g**) Longitudinal section of posterior cardinal vein (PCV) from E10.5 *Edg1*$^{+/-}$ embryo. LacZ staining is seen in arterial endothelial cells (AEC) but not in venous endothelial cells (VEC). (**h**) Transverse section of dorsal aorta from E12.5 *Edg1*$^{+/-}$ embryo. Vascular ECs and VSMCs are stained. EC, endothelial cell; VSMC, vascular smooth muscle cell. Scale bars = 50 μm.

Figure 2
Phenotype of *Edg1*$^{-/-}$ embryos and normal vascular network in the *Edg1*$^{-/-}$ embryos. Photomicrographs of E12.5 and E13.5 embryos with the amnion, yolk sac, and placenta intact (**a** and **c**), or with extraembryonic membranes removed (**b** and **d**). *Edg1*$^{-/-}$ embryos show normal yolk sac vasculature but with less blood (arrows). Yolk sacs of *Edg1*$^{-/-}$ embryos display progressive edema. E12.5 *Edg1*$^{-/-}$ embryo shows intraembryonic hemorrhages in the body and limbs. FL, front limb; HL, hind limb. E13.5 *Edg1*$^{-/-}$ embryo demonstrates severe intraembryonic hemorrhages and edema. Both E12.5 and E13.5 *Edg1*$^{-/-}$ embryos display pericardial cavity (PCC) edema. (**e**–**h**) E12.5 wild-type and *Edg1*$^{-/-}$ embryos were stained with an anti-CD34 mAb and visualized by low-power (**e** and **f**) or higher-power (**g** and **h**) magnification. Note the normal vascular patterning, capillary plexus, and capillary sprouting (black arrowheads) in the *Edg1*$^{-/-}$ embryos. Small blood vessels in the forebrain of *Edg1*$^{-/-}$ embryos are slightly dilated (white arrowheads).



throughout the body of *Edg1*$^{-/-}$ embryos (Figure 3d). No *Edg1*$^{-/-}$ embryos survived beyond E14.5 (Table 1).

Normal vasculogenesis in Edg1$^{-/-}$ *embryos.* To define the vascular system in the *Edg1*$^{-/-}$ embryos, the morphology of the vasculature was characterized by whole-mount immunohistochemical staining using mAb's against markers for endothelial cells. Antibodies to CD34 (Figure 2, e and f) and platelet endothelial cell adhesion molecule-1 (PECAM-1) (data not shown) revealed a substantially normal arborized vascular network both in the mutants and age-matched control embryos. High magnification views showed capillary sprouts in the head of mutant embryos (Figure 2, g and h, black arrowhead). However, the small vessels in the forebrain of mutant embryos appeared dilated and stained darker than controls with antibodies against PECAM-1 and CD34 (white arrowheads).

Because SPP signaling through Edg-1 was shown to be involved in adherens junction assembly in HUVEC cells in vitro (24), we investigated the expression of VE-cadherin, P-cadherin, E-cadherin, N-cadherin, and PECAM-1 in *Edg1*$^{-/-}$ embryos by using whole-mount immunohistochemistry and Western blot analysis. No obvious difference was observed in expression patterns between mutant and control embryos. Disruption of VE-cadherin function causes increased endothelial apoptosis (25); however, there was no apparent enhancement of endothelial cell death in E12.5 *Edg1*$^{-/-}$ embryos as determined by TUNEL staining (data not shown).

Because *Edg1* is highly expressed in vascular endothelial cells, endothelial cell proliferation and differentiation signaling factors, including *VEGF, Flt-1, Flk-1, Tie-1, Tie-2, Ang-1,* and *Ang-2,* were analyzed by Western blot and RT-PCR methods. All of these genes were expressed in *Edg1*$^{-/-}$ embryos at levels similar to those of control embryos (data not shown). Thus the expression of genes required for early differentiation and assembly of endothelial cells into the vascular network was not impaired in the *Edg1*$^{-/-}$ embryos. Our data indicate that in *Edg1*$^{-/-}$ embryos, vasculogenesis

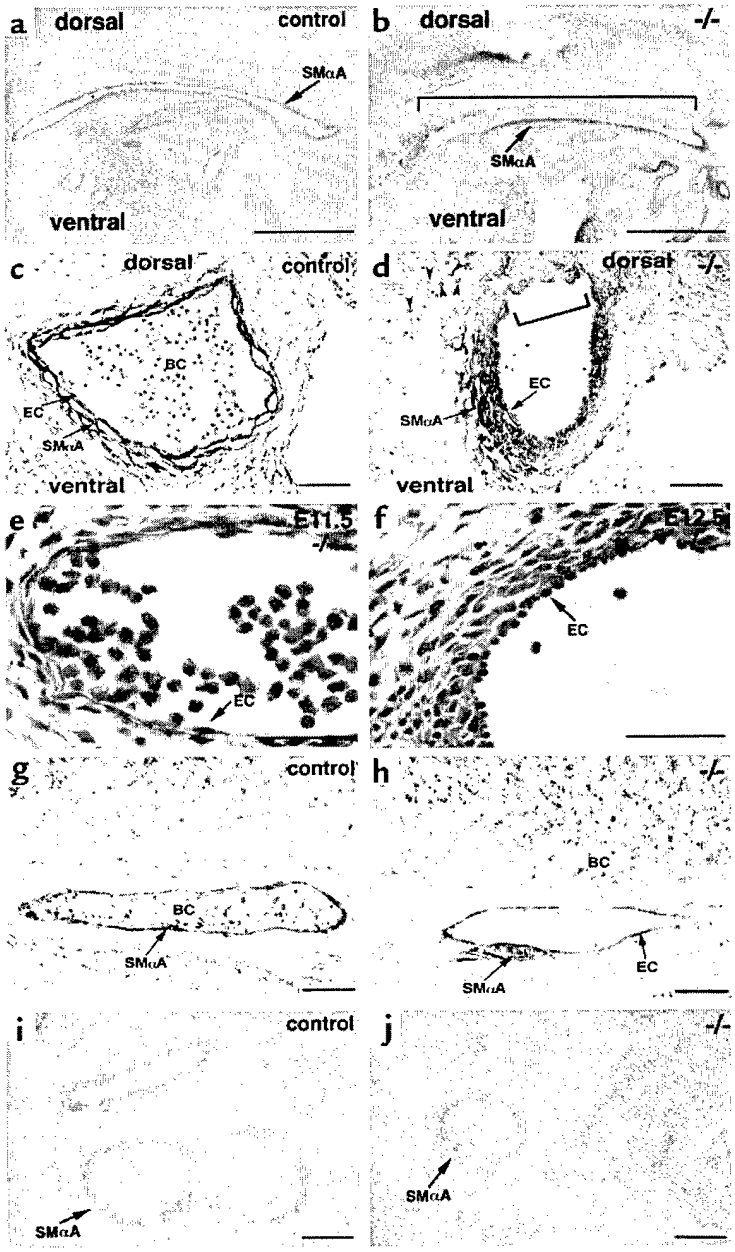


Figure 3
Vascular smooth muscle defects in the *Edg1*$^{-/-}$ embryos. (**a** and **b**) Aortae of E12.5 embryos, sectioned longitudinally and stained with anti-SMαA antibody. Note the lack of SMαA-positive smooth muscle cells (bracket) on the dorsal side of aorta in the *Edg1*$^{-/-}$ embryo. (**c** and **d**) Transverse sections of aortae from E12.5 embryos stained with anti-SMαA. Smooth muscle cells have accumulated at the ventral site of the aorta in the *Edg1*$^{-/-}$ embryo. Note the discontinuous endothelial cell (EC) layer (bracket) in the *Edg1*$^{-/-}$ embryo. Many blood cells have leaked out to the surrounding tissues in the *Edg1*$^{-/-}$ embryo (arrowheads). BC, blood cells. (**e** and **f**) H&E staining of aorta from E11.5 and 12.5 *Edg1*$^{-/-}$ embryos. Arrows point to ECs. Note their normal, flattened morphology in **e** and abnormal, cuboidal morphology in **f**. (**g** and **h**) Cranial arteries from E12.5 embryos stained with anti-SMαA antibody. Note the clustering of smooth muscle cells and nearly naked endothelial tube from the *Edg1*$^{-/-}$ embryo. (**i** and **j**) Sections of intestine from E12.5 embryos stained with anti-SMαA. Note that that coverage of intestine by smooth muscle is similar in control and mutant embryos. Scale bars = 1 mm (**a** and **b**); 50 μm (**c** and **d**); 50 μm (**e** and **f**); 50 μm (**g** and **h**); 500 μm (**i** and **j**).

and the phase of angiogenesis that entails vessel sprouting and penetration had occurred.

Vascular smooth muscle defects in Edg1$^{-/-}$ *embryos.* After the initial formation of the vascular plexus, vessels mature by the stabilization of the endothelial vascular network through a recruitment and differentiation process that ultimately results in the investment of vessel walls with mural cells (26).

Vascular smooth muscle cells (VSMCs) first appear on the ventral side of the aorta in E10.5 embryos, followed by migration to the dorsum (27). By E11.5, the aorta is completely enveloped by VSMCs (27). To assess this aspect of vessel development in the *Edg1* mutant embryos, VSMCs were identified using an antibody to SMαA. In longitudinal sections of E12.5 control embryos stained with anti-SMαA, the dorsal aortae were found to be completely surrounded by VSMCs (Figure 3a). The aortae of *Edg1*$^{-/-}$ mice were strikingly different. SMαA-positive VSMCs were present on the ventral surface; however, VSMCs were deficient along the entire length of the dorsal surface examined (Figure 3b).

Transverse sections of aortae from control embryos showed two to three layers of VSMCs surrounding the

vessel (Figure 3c). In contrast, similar sections from *Edg1*$^{-/-}$ embryos showed that the aortae were covered only ventrally by poorly organized SMαA-expressing cells (Figure 3d). SMαA-expressing cells were not found on the dorsal side of the mutant aortae. These results suggested initial recruitment and differentiation had taken place to produce VSMCs on the ventral side of the mutant aorta, but that the process leading to the complete envelopment of the vessel was defective. Endothelial cell morphology appeared normal in E11.5 embryos, before the onset of bleeding (Figure 3e). In the E12.5 embryos, after the onset of bleeding, the dorsal aortic surface (uncovered by VSMCs) appeared abnormal and discontinuous (Figure 3, d and f).

In E12.5 control embryos, the majority of medium-sized arteries, identified by association with SMαA-positive VSMCs, were surrounded by a continuous layer of VSMCs (Figure 3g). Only rare vessels were found with an incomplete covering by VSMCs. In contrast, a substantial faction of intracerebral arteries in the mutant embryos displayed a discontinuous or patchy covering by VSMCs (Figure 3h). Bleeding from these arteries was apparent by the presence of blood cells in the surrounding tissue space (Figure 3h).

The muscular layers in the gastrointestinal tract (Figure 3, i and j) and bronchial tree (data not shown) were well developed in the mutant embryos, indicating that there was not a generalized defect in smooth muscle.

The blood vessel defects in the *Edg1*$^{-/-}$ embryos were further analyzed by electron microscopy. Small blood vessels from the limb of E12.5 *Edg1*$^{-/-}$ embryos illustrated a marked reduction of VSMCs/pericytes adjacent to the endothelial cells (Figure 4, a and b). The endothelial cell body was very thin, and in some areas, fragmented (data not shown). Intracerebral capillaries of mutant embryos appeared without associated microvascular pericytes (Figure 4, c and d). The endothelial cell nuclei of the mutant capillaries were abnormally rounded and enlarged. The areas surrounding these vessels were generally much less densely packed with cells. Mutant blood vessels contained normal-appearing, electron-dense interendothelial junctions (Figure 4, e and f), suggesting that endothelial cell-cell junctions formation occurred in the absence of Edg-1. Given that PDGF-B and its receptor β are critical for the investment of capillaries with pericytes (28–30), we determined their expression in mutant embryos. We found that in *Edg1*$^{-/-}$ embryos PDGF-B expression was normal as determined by Western analysis and that PDGF-receptor β was highly expressed in mesenchymal cells by immunohistochemical analysis (data not shown). Mice deficient in the transcription factor LKLF also show marked reductions in VSMCs and pericytes around vessels (31). RT-PCR of E12.5 *Edg1*$^{-/-}$ RNA indicated that LKLF expression was similar to control levels (data not shown).

SPP induced migration in Edg1$^{-/-}$ *fibroblasts.* Our analysis of *Edg1* embryos demonstrated a defect in blood vessel maturation that appeared to involve the ability of mural cells to properly organize and reinforce endothelial walls. Recently, Edg-1 has been implicated as the mediator of an SPP-induced migration response in different cell types (32–35). We investigated whether cells derived from the mutant embryos were defective in their migration response to SPP.

Fibroblasts were obtained from control and *Edg1*$^{-/-}$ embryos. RT-PCR revealed that wild-type fibroblasts expressed transcripts for *Edg1*, *-3,* and *-5* genes (Figure 5a). *Edg1*$^{-/-}$ fibroblasts, as expected, were devoid of the authentic *Edg1* transcript but contained transcripts for *Edg3* and *-5*. The mutant cells adhered normally to tissue culture plates and exhibited a normal mitogenic response to SPP (data not shown).

As shown in Figure 5b, 100 nM SPP induced a significant increase in the chemotaxis of wild-type fibroblasts. In contrast, the *Edg1*$^{-/-}$ fibroblasts did not display a significant migratory response to SPP, proving that Edg-1 is required for SPP-induced migration in these cells. Because Rac activation, which has been shown to be stimulated by SPP (24), is critical for cell migration responses (36), we compared the effect of

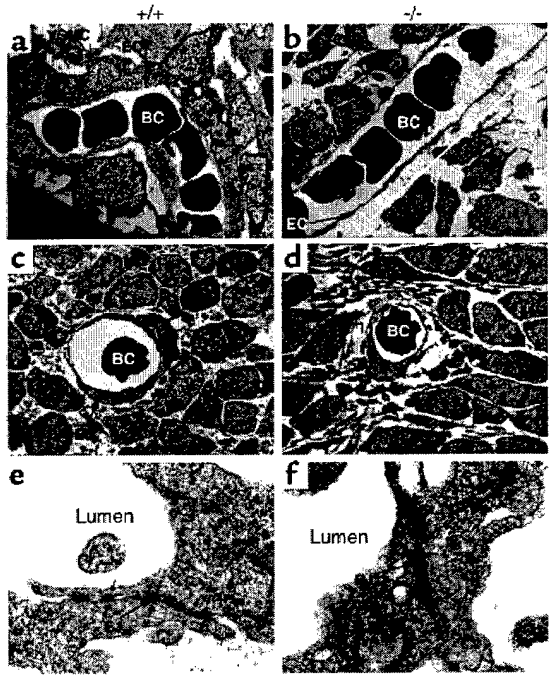
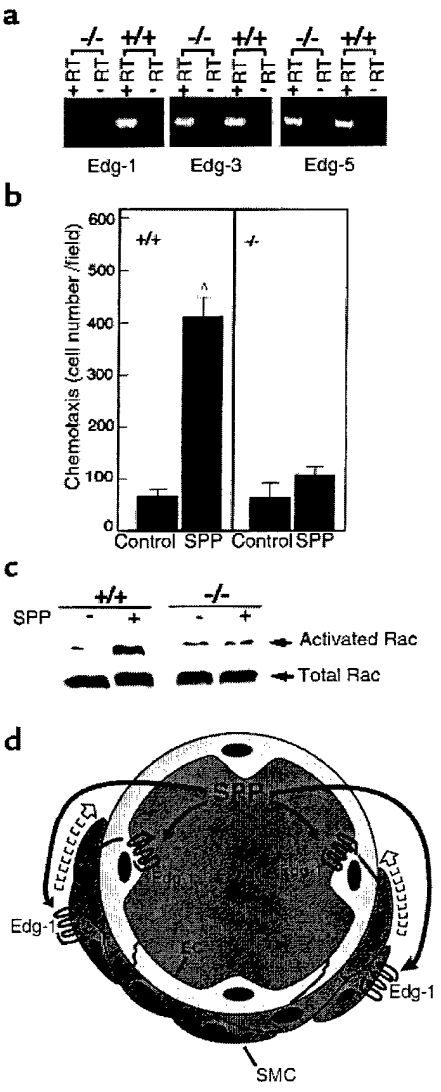


Figure 4
Reduced VSMCs and pericytes in the *Edg1*$^{-/-}$ vessels. (**a–d**) EM microscopic analyses of representative small blood vessels from the limb (**a** and **b**) and brain capillaries (**c** and **d**) from E12.5 wild-type and *Edg1*$^{-/-}$ embryos. Reduced number of VSMCs (bracket in **b**) and the lack of capillary pericytes (PC) were found in the *Edg1*$^{-/-}$ embryos. Notice the abnormally rounded EC nucleus in the *Edg1*$^{-/-}$ capillary (**d**). (**e** and **f**) EC junctions (arrows) in wild-type and mutant embryos. Note the normal EC junction (EJ) in the *Edg1*$^{-/-}$ embryo (**f**). BC, blood cell. ×2,000 (**a–d**); ×50,000 (**e** and **f**).

Figure 5
Migration and Rac activation defects in *Edg1*$^{-/-}$ embryonic fibroblasts. (**a**) RT-PCR analysis of *Edg1, -3, and -5* expression was carried out with total RNA isolated from *Edg1*$^{+/+}$, *Edg1*$^{+/-}$, and *Edg1*$^{-/-}$ embryonic fibroblasts. (**b**) SPP chemotactic responses of embryonic fibroblasts. Serum-starved *Edg1*$^{+/+}$ and *Edg1*$^{-/-}$ fibroblasts were allowed to migrate toward a gradient produced by SPP (100 nM). Control (Cont.) indicates medium without serum was used as the chemoattractant. Chemotaxis was measured as described in Methods. Data are means ± SD of triplicate determinations. ^A^Statistically significant difference compared with the control, determined by Student's *t* test ($P < 0.01$). (**c**) Rac activation in fibroblasts. *Edg1*$^{+/+}$ and *Edg1*$^{-/-}$ fibroblasts were serum-starved and then treated with SPP for 5 minutes. The cell lysates were used both for affinity precipitation with the PAK-1–conjugated agarose to pull down activated, GTP-bound Rac (top panel) and without fractionation to determine total Rac levels (bottom panel) by SDS-PAGE and immunoblotting. (**d**) Model of Edg-1 functions in blood vessel development. The *Edg1* knockout demonstrates that Edg-1 is essential for vascular maturation by impairing the recruitment of smooth muscle cells to vessel walls. SPP, found in blood, may directly stimulate Edg-1 on VSMCs, facilitating their migration to vessels walls. In a second mechanism, which does not exclude the first, SPP could stimulate Edg-1 expressed on endothelial cells, which in turn recruit may VSMCs. EC, endothelial cell; SMC, smooth muscle cell.



SPP on Rac activation in wild-type and *Edg1*$^{-/-}$ fibroblasts (Figure 5c). In wild-type fibroblasts, SPP treatment resulted in a substantial increase in the amount of activated Rac. By contrast, no increase in activated Rac could be detected after SPP treatment of *Edg1*$^{-/-}$ fibroblasts, demonstrating that Edg-1 is required for the SPP induction of activated Rac.

Discussion

A number of receptor-mediated signaling pathways have been identified that coordinate the stages of blood vessel formation. Disruption of the genes encoding these receptors and ligands in mice has been instrumental in defining their roles (reviewed in refs. 26, 37, 38). Vasculogenesis is dependent on VEGF and its receptor tyrosine kinases, Flk-1 and Flt-1, expressed in endothelial cells (39–42). *VEGF, Flk-1,* and *Flt-1* knockout mice die between E8.5 and E9.5 as a result of defects in the formation of the primitive vasculature. Angiogenesis and vascular remodeling require the Tie-2 receptor tyrosine kinase on endothelial cells and its ligand, angiopoietin-1. Without Tie-2, mice have normal vasculogenesis but defective vessel sprouting, branching, and remodeling; they die at E10.5. Mice devoid of angiopoietin-1 have a similar phenotype (43–45).

Signaling pathways have also been implicated in the recruitment and differentiation of mural cells during vessel maturation. PDGF-B and its receptor-β have been shown to be essential for the recruitment of mesenchymally derived mural cell precursors to vessel walls (30, 46). Disruption of the *PDGF-B* or *PDGF receptor-β* genes in mice leads to lethal hemorrhage and edema in the perinatal stage owing to a lack of microvasculature pericytes (28–30). TGF-β1 induces differentiation of VSMCs (47) after their recruitment to endothelial walls (48, 49). The endothelial TGF-β1 binding protein, endoglin, and its downstream signaling molecule, SMAD5, both important in the TGFβ1 signaling pathway, also have essential roles in VSMC differentiation (50, 51). *Endoglin-* and *Smad5*-deficient mice die between E10.5 and E11.5 with a lack of VSMCs around major vessels.

These studies point to the critical roles played by receptor tyrosine kinases during vascular development. In contrast, the involvement of G-protein–coupled signaling pathways have not been as well characterized during development. Such pathways are important because disruption of the $G_{\alpha,13}$ gene results in defects in embryonic vasculature formation, presumably due to a migration defect (52), However, upstream receptors involved had not been defined.

Role of Edg-1 during early vascular system development. We found that *Edg1* was highly expressed in the cardiovas-

cular system during early embryonic development. Vascular endothelial cells expressed *Edg1* at relatively high levels, although expression was almost exclusively found in the endothelial cells of arteries rather than of veins. A low but detectable expression was also found in VSMCs surrounding the aorta. Expression of *Edg1* was prominent in cardiomyocytes, although no gross abnormalities were detected in the developing heart. In addition to the vascular system, *Edg1* was found highly expressed in the developing central nervous system as has been shown previously (16, 18).

Severe bleeding caused lethality in *Edg1*$^{-/-}$ embryos between E12.5 and E14.5. However, the mutant embryos showed a substantially normal blood vessel network when stained with antibodies to markers specific for differentiated endothelial cells such as PECAM-1 and CD34. The expression of genes known to be important for vasculogenesis and angiogenesis was not measurably affected in *Edg1*$^{-/-}$ embryos. These genes included *VEGF, Flk-1, Flt-1, Ang-1,* and *Tie-2*. Each phenotype of these knockout mice is quite distinct from that of the *Edg1*$^{-/-}$ mice, which develop a relatively normal appearing vascular network and die between E12.5 and E14.5. These results indicate that *Edg1* is dispensable for vascular endothelial cell differentiation, proliferation, migration, and tube formation during vasculogenesis and for the stage of angiogenesis involving vessel sprouting and branching. The morphology and viability of endothelial cells in the mutants appeared normal until the onset of severe bleeding, suggesting that the morphology changes were secondary to the lack of supporting VSMCs and subsequent disruption of the vessels in the mutant embryos.

Although SPP signaling through Edg-1 has been shown to regulate adherens junction formation in vitro in human endothelial cells, we found no evidence of aberrant endothelial junctions in *Edg1* mutant mice during development. Ultrastructural analysis of mutant endothelial cells revealed normal appearing cell-cell junctions. The phenotype of the *Edg1* mutant embryos was also not in keeping with significant defects in endothelial cell junctions. Recently, it was shown that VE-cadherin, an important component of adherens junctions, controls an endothelial cell survival pathway through its intracellular interaction with β-catenin (25). Disruption of this pathway in mice resulted in impaired angiogenesis, increased endothelial apoptosis, and embryonic death by E9.5. The *Edg1* mutant mice exhibited none of these characteristics. Thus the formation of functional endothelial junctions apparently proceeds normally during early development in the absence of Edg-1. This could indicate that there is functional redundancy among members of the Edg family for this process and that other Edg proteins may substitute for Edg-1. It may also indicate that the SPP-Edg regulation of adherens junction assembly is not required for blood vessel formation during development. However, this would not preclude a role for the pathway during angiogenesis in the adult.

Functions of Edg-1 during vessel maturation. In dorsal aorta, VSMC investment is initiated on the ventral side with a condensation of SMαA-positive cells. The recruitment process continues dorsally until VSMCs have completely enveloped the endothelial tube (27). We found that the aortae of wild-type and heterozygous embryos were surrounded by several layers of elongated VSMCs. The aortae in the *Edg1*$^{-/-}$ embryos were strikingly abnormal in VSMC investment. Aortic sections demonstrated the presence of multiple layers of SMαA-positive cells, but only at the ventral surface. These results suggest that the defect in VSMC investment of vessel walls in *Edg1* mutant embryos was expressed after the initial VSMC recruitment to the ventral aortic surface has taken place. This vessel abnormality was distinct from that observed in mice deficient in endoglin, the TGF-β binding protein on endothelial cells, and in SMAD5, the TGF-β1 signaling molecule. Both of these proteins are required for VSMC differentiation; the respective knockout mice have severely defective VSMC development with almost no SMαA-positive cells around vessels (50, 51).

In addition to a defect in mural cell recruitment in dorsal aorta, the *Edg1*$^{-/-}$ mice exhibited defects in the smaller vessels and in the microvasculature. By electron microscopy, we found evidence of a lack of pericytes associated with capillaries. When pericytes are deficient as in *PDGF-B*$^{-/-}$ mice, dilated microvessels develop that are prone to rupture (53). Similarly, in the *Edg1*$^{-/-}$ mice, dilation of small cranial vessels and bleeding were evident.

How does Edg-1 so dramatically influence the recruitment of VSMCs to vessels during development? With fibroblasts from the *Edg1* mutant mice, we found that in the absence of Edg-1, the SPP-induced activation of Rac seen in wild-type cells did not occur. Rac is a key regulator of the actin cytoskeleton and of associated activities such as cell motility (36, 54). Thus, the *Edg1*$^{-/-}$ fibroblasts, without this signaling pathway, were unable to mount a migration response to SPP. This signaling pathway may also operate in VSMCs, and its disruption could be responsible for the defect in vascular maturation seen in the *Edg1*$^{-/-}$ mice. Consistent with this hypothesis, we found that Edg-1 mediates migration of normal VSMCs toward SPP (data not shown). SPP is abundantly stored in platelets and secreted after stimulation (55). Other blood cells, including erythrocytes, neutrophils, and mononuclear cells, produce and secrete SPP constitutively (56), resulting in significant SPP levels in blood. During maturation of the dorsal aorta and possibly other vessels, plasma containing SPP could escape from immature, leaky vessels and act as a signal to recruit VSMCs that are differentiating in the proximity of vessel walls (Figure 5d).

Recently, it was shown that the zebra fish gene, *mil*, encodes an SPP-binding, G protein–coupled receptor of the Edg family that directs the migration of heart precursors to the midline during embryonic development (57). Interestingly, *mil* does not function in the

migrating precursor cells, but in the paraxial cells at the midline, presumably by creating a permissive environment for migration. Sequence and functional similarities suggest that *mil* may be the ortholog of the mammalian *Edg5* gene. However, the indirect influence of *mil* on migrating cells, as well as the high level of expression of *Edg1* in arterial endothelial cells, raises the possibility that Edg-1 stimulation on endothelial cells may regulate the recruitment of VSMCs (Figure 5d). This might occur via the upregulation of adhesion molecules on endothelial cells, or by stimulating the secretion of recruitment factors for VSMCs. Further studies using tissue-specific knockouts of *Edg1* will be required to address precisely how Edg-1 functions. Nevertheless, our data demonstrate an indispensable role of *Edg1* in vascular maturation and, together with the results showing *mil* is essential for heart organogenesis, indicate that different members of the Edg receptor family regulate distinct aspects of cardiovascular development through sphingolipid signaling pathways.

In summary, we have uncovered a unique and vital role for the G protein–coupled receptor, Edg-1, in blood vessel formation during development. The SPP-Edg signaling pathway may also have important functions in adult vascular biology. SPP stimulation of Edg receptors on endothelial cells in vitro results in proliferation, migration, and tube formation (24, 32, 58), all requisite for the angiogenic process. Thus, the release of SPP after platelet activation, with subsequent stimulation of the Edg receptors on both endothelial cells and VSMCs, may drive blood vessel formation during wound healing and solid tumor growth (59, 60). If so, the SPP-Edg signaling pathway is a potential target for therapeutic manipulation during these and other processes that are dependent on angiogenesis.

Acknowledgments

We thank J. Van Brocklyn for help in the early stages of this project and A. Howard for producing the figures.

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Role of the Sphingosine-1-Phosphate Receptor EDG-1 in PDGF-Induced Cell Motility

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EDG-1 is a heterotrimeric guanine nucleotide binding protein-coupled receptor (GPCR) for sphingosine-1-phosphate (SPP). Cell migration toward platelet-derived growth factor (PDGF), which stimulates sphingosine kinase and increases intracellular SPP, was dependent on expression of EDG-1. Deletion of EDG-1 or inhibition of sphingosine kinase suppressed chemotaxis toward PDGF and also activation of the small guanosine triphosphatase Rac, which is essential for protrusion of lamellipodia and forward movement. Moreover, PDGF activated EDG-1 as measured by translocation of β-arrestin and phosphorylation of EDG-1. Our results reveal a role for receptor cross-communication in which activation of a GPCR by a receptor tyrosine kinase is critical for cell motility.

Interest in SPP has accelerated recently with the discovery that it is the extracellular ligand for EDG-1, -3, -5, -6, and -8 (1). Although the biological functions of these GPCRs have not been completely elucidated, EDG-1 is implicated in cell migration, angiogenesis, and vascular maturation (2, 3, 4). Disruption of the *edg-1* gene by homologous recombination in mice resulted in massive intra-embryonic hemorrhaging and intrauterine death caused by incomplete vascular maturation resulting from a failure of mural cells - vascular smooth muscle cells and pericytes - to migrate to arteries and capillaries and properly reinforce them (4). Disruption of the *PDGF-BB* or *PDGFR-β* genes in mice resulted in a similar lethal phenotype (5, 6). Since in many different cell types, PDGF stimulates sphingosine kinase leading to an accumulation of intracellular SPP (1, 7), we speculated that interplay between PDGF and SPP-EDG-1 signals might be required for cell migratory responses. In this study, we found that activation of EDG-1 by the PDGFR plays a crucial role in regulating cell motility. The results reveal a new paradigm for communication between tyrosine kinase receptors and GPCRs.

Human embryonic kidney (HEK) 293 cells, which only express EDG-3 and EDG-5, did not migrate toward SPP unless EDG-1 was expressed (2). EDG-1 overexpression also stimulated migration of HEK 293 cells toward PDGF-BB (Fig. 1A), whereas migratory responses to serum and fibronectin were unaffected (Fig. 1A). Conversely, migration of mouse embryonic fibroblasts (MEFs), which express transcripts for EDG-1, EDG-3, and EDG-5, but not EDG-6 or EDG-8 (4), toward PDGF-BB was reduced when *edg-1* was deleted (Fig. 1B). A smaller effect on migration toward serum was observed in these mutant fibroblasts and migration toward

fibronectin was unaffected (Fig. 1B), indicating that *edg-1* deletion does not disrupt all essential mechanisms of directed cell movement.

Because the migration of smooth muscle cells appears to be aberrant in EDG-1 knockout mice (4), we also examined the role of EDG-1 in PDGF-directed migration of human aortic smooth muscle cells (ASMC). Reduction of EDG-1 expression in ASMC (which endogenously express EDG-1, -3, and -5) by EDG-1 antisense phosphothioate oligonucleotide (3), not only eliminated migration toward SPP but also reduced migration toward PDGF, but not serum (Fig. 1C). These results suggest that the loss of EDG-1 results in motility defects toward PDGF in diverse cell types. Dysfunctional migration of EDG-1 -/- cells toward PDGF links this phenotype (4) to the PDGF-BB and β-PDGFR knockout phenotypes (5, 6) at the final steps of vasculogenesis (4), underscoring the importance of cross-communication between PDGFR and EDG-1 in vascular maturation.

Like it does in many other cell types (1, 7), PDGF-BB stimulated sphingosine kinase activity in wild-type MEFs and had an even greater stimulatory effect in *edg*-1-deleted fibroblasts (Fig. 2A). To investigate whether SPP generated in response to PDGF might be involved in PDGF-mediated chemotaxis, we used N,N-dimethylsphingosine (DMS), a competitive inhibitor of sphingosine kinase (8). DMS inhibited PDGF-directed chemotaxis of wild-type MEFs (Fig. 2B), but did not reduce PDGF-stimulated receptor tyrosine phosphorylation (Fig. 2C). In agreement with its inability to interfere with binding of SPP to EDG-1 and its activation (9), DMS did not significantly affect chemotaxis of cells toward a gradient of SPP. Similarly, DMS also blocked formation of SPP and inhibited PDGF-directed chemotaxis of HEK 293 cells

overexpressing EDG-1 (Fig. 2D). As these results indicate potential crosstalk between PDGF and EDG-1 signaling, and EDG-1 is mainly coupled to G_i (9), cells were pretreated with pertussis toxin to inactivate G_i, which suppressed PDGF-induced chemotaxis of both wild-type MEFs (Fig. 2B) and HEK 293 cells overexpressing EDG-1 (Fig. 2D).

EDG-1 thus appears to be necessary for PDGF-mediated chemotaxis. Therefore, we determined whether PDGF signaling might activate EDG-1 by regulating sphingosine kinase activity and accumulation of SPP, which in turn activates EDG-1. β-Arrestins are cytosolic adapter proteins that bind with high affinity to agonist-activated, phosphorylated GPCRs to terminate receptor G protein coupling and also play other roles in receptor endocytosis (10, 11) and initiation of a second wave of signaling (12, 13). SPP promoted rapid redistribution of β-arrestin2 tagged with green fluorescent protein from the cytoplasm to the plasma membrane only in EDG-1-expressing HEK 293 cells (Fig. 3A.b). Treatment of cells overexpressing both EDG-1 and sphingosine kinase type 1 (SPHK1) with PDGF and sphingosine to increase intracellular SPP, induced translocation of β-arrestin2 to the plasma membrane (Fig. 3B.c), as did exposure of cells to exogenous SPP (Fig. 3B.b). Availability of sphingosine is a limiting factor influencing levels of cellularly generated SPP (14). Thus, sphingosine-induced translocation of β-arrestin was dependent on overexpression of SPHK1 (note the lack of translocation in Fig. 3A.c). Although treatment with PDGF, sphingosine alone, or transfection with SPHK1 stimulated production of SPP by 2-, 6-, and 10-fold, respectively (Fig. 3C), these concentrations of SPP did not result in significant translocation of β-arrestin. In contrast,

translocation was readily observed after treatment of SPHK1-expressing HEK 293 cells with a high concentration of sphingosine (Fig. 3B.d) or with lower concentrations together with PDGF (Fig. 3B.c), which increased SPP levels by 60- and 30-fold, respectively. To further substantiate that PDGF activates EDG-1, we examined whether PDGF itself induces phosphorylation of EDG-1, as ligand-induced phosphorylation of GPCRs is required for β-arrestin binding (10, 12). To enhance sensitivity of detection, HEK 293 cells were co-transfected with expression plasmids encoding Flag epitope-tagged EDG-1 and β-PDGFR, labeled in situ with $^{32}P_i$, and EDG-1 was immunoprecipitated with anti-Flag antibody. As did SPP (15), PDGF increased phosphorylation of EDG-1 in these cells (Fig. 3D), whereas no phosphorylation could be detected in vector transfected cells (16).

Although these results suggest that endogenously generated SPP can activate EDG-1 and consequent translocation of β-arrestin, no significant release of SPP into the extracellular media could be detected by mass measurements (< 0.4 nM), even after treatment of SPHK1-expressing HEK 293 cells with PDGF-BB and sphingosine to increase SPP. To examine the possibility that amounts of SPP below our mass detection limits may in fact be secreted into the vicinity of EDG-1 and activate it, SPHK1-expressing cells were transfected with β-arrestin2 fused to red fluorescent protein at the N-terminus (red) to differentiate them from β-arrestin2-GFP (green) transfectants. The β-arrestin2-RFP transfectants were treated with 10 μM sphingosine to maximally increase intracellular SPP. Conditioned medium from these SPP-producing cells did not induce translocation of β-arrestin2-GFP in EDG-1 transfected cells (Fig. 3E.a). Nevertheless, co-culturing of "red" SPP-producing cells with "green"

EDG-1-transfected cells induced translocation of β-arrestin2-GFP to the plasma membrane on adjacent cells (Fig. 3E.b) and on distant cells (Fig. 3E.c). These results suggest that endogenously generated SPP can activate EDG-1 in an autocrine or paracrine manner.

Although deletion of EDG-1 or uncoupling G_i inhibited PDGF-directed motility, there were no significant differences in PDGFR expression or PDGF-stimulated receptor tyrosine phosphorylation in wild-type compared to EDG-1 null fibroblasts (Fig. 4A). Rac, a member of the Rho family of small GTPases (Rac, Cdc42, and Rho), plays a critical role in cell motility by regulating formation of new lamellipodial protrusions at the leading edge (17). PDGF-BB rapidly activated Rac (Fig. 4B), but not Cdc42, in wild-type fibroblasts. Deletion of *edg-1,* or inhibition of sphingosine kinase in wild-type MEFs, decreased Rac activation induced by PDGF. These results suggest that Rac may participate in integration of PDGFR and EDG-1 signaling to promote cell migration and that the SPP signaling pathway might be important to amplify activation of Rac.

Various agonists for GPCRs can activate growth factor tyrosine kinase receptors in the absence of added growth factors (18). While this type of cross communication is important for regulation of cell growth (18), our results suggest that cell motility is regulated by a reciprocal mechanism of receptor crosstalk. Thus, a tantalizing notion is that spatially and temporally localized generation of SPP by activation of sphingosine kinase in response to PDGF results in restricted activation of the GPCR EDG-1 that in turn activates Rac (Fig. 4C). Rac may then amplify the initial receptor signals (19), thus creating a positive feedback loop at the leading edge of the cell.

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21. Supported by NIH Grant GM43880 to SS and in part by NIH Grants CA61774 to SS and HL-61365 to LSB and DAMD17-99-1-9188 to JPH and 0020255U to HR and NS19576 to MGC. We thank Drs. Richard Proia and Yujing Liu for generously providing us with MEFs and for helpful discussions.

FIGURE LEGENDS

Fig. 1. Requirement of EDG-1 for PDGF-induced migration. (A) Enhanced chemotaxis toward PDGF in cells overexpressing EDG-1. HEK 293 cells stably transfected with vector (open bars) or EDG-1 (filled bars) were allowed to migrate toward PDGF-BB (20 ng/ml), SPP (100 nM), serum (FBS, 20 %), or fibronectin (FN, 10 µg/ml). Chemotaxis was measured in a modified Boyden chamber assay (2). The average number of cells in four random fields was determined and is presented as the average ± S.D. of three individual wells. Similar results were obtained in at least three independent experiments and statistically different groups are indicated by asterisk ($p < 0.05$ by analysis of variance) **(B)** Chemotaxis toward PDGF is markedly reduced by *edg-1* disruption. Wild type (open bars) and EDG-1 -/- (filled bars) MEFs were allowed to migrate toward PDGF-BB, SPP, serum or FN as in A. **(C)** Dependence of

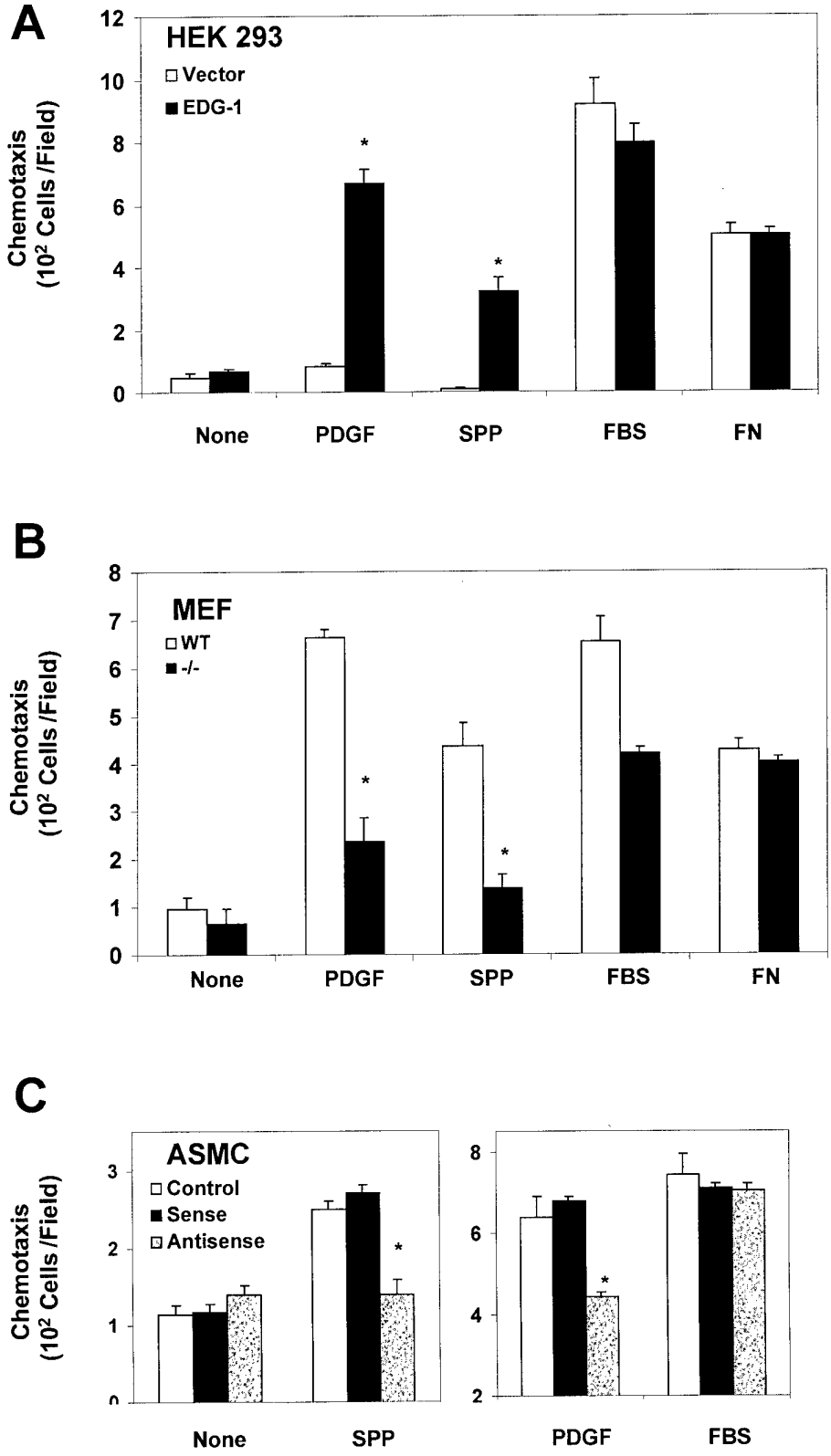
chemotaxis of ASMC toward PDGF and SPP on EDG-1 expression. Human ASMC were transfected without (open bars) or with 400 nM phosphothioate oligonucleotides [EDG-1 sense (filled bars), 5'-ATG GGG CCC ACC AGC GTC-3'; EDG-1 antisense (gray bars), 5'-GAC GCT GGT GGG CCC CAT-3'] using Lipofectamine (Life Technologies, Gaithersburg, MD) (3) and after 24 hours, allowed to migrate toward SPP (100 nM), PDGF (20 ng/ml), or FBS (20%). Reverse transcriptase-polymerase chain reaction analysis showed that EDG-1 mRNA expression was almost completely eliminated without affecting EDG-3 and EDG-5 expression.

Fig. 2. Requirement of sphingosine kinase for PDGF-induced motility. (A) Stimulation of sphingosine kinase by PDGF in wild-type and EDG-1 -/- MEFs. Wild-type and EDG-1 -/- MEFs were treated with PDGF-BB (20 ng/ml) for the indicated times and sphingosine kinase activity was measured. Basal activity was 114 ± 5 and 133 ± 2 pmol/min/mg for wild-type and -/- fibroblasts, respectively. **(B, D)** Inhibition of chemotaxis toward PDGF by the sphingosine kinase inhibitor DMS or pertussis toxin. Wild-type MEFs **(B)** or HEK 293 cells transfected with vector or EDG-1 **(D)** were treated without (Control) or with pertussis toxin (PTX, 200 ng/ml, 2 hours), or DMS (10 μM, 20 min), then allowed to migrate towards the indicated concentrations of PDGF-BB and chemotaxis was measured. (**C**) Lack of effect of DMS on tyrosine phosphorylation induced by PDGF. Wild-type MEFs were serum-starved for 24 hours, and then treated without or with DMS (10 μM) for 20 min, prior to stimulation with PDGF-BB (60 ng/ml) for 5 min. Equal amounts of cell lysate proteins were analyzed by Western blotting using anti-phosphotyrosine antibody.

Fig. 3. SPP produced intracellularly can act in an autocrine or paracrine fashion to activate EDG-1. **(A)** β-Arrestin translocation in HEK 293 cells co-transfected with expression plasmids encoding β-arrestin2-GFP and EDG-1. β-Arrestin2-GFP fluorescence was visualized (10) after treatment with vehicle (**a**), 1 μM SPP (**b**) or 10 μM sphingosine (**c**). (**B**) Translocation of β–arrestin in HEK 293 cells co-transfected with β-arrestin2-GFP, EDG-1, and SPHK1. β-arrestin2-GFP fluorescence was visualized after treatment with vehicle (**a**), 1 μM SPP (**b**), 2.5 μM sphingosine and 20 ng/ml PDGF-BB (**c**) or 10 μM sphingosine (**d**). **(C)** Cellular levels of SPP. Levels of SPP were measured in vector transfected (open bar) and in SPHK1 transfected (filled bars) HEK 293 cells after treatment with 2.5 μM sphingosine, 20 ng/ml PDGF-BB, or both for 10 min (20). **(D)** PDGF-induced EDG-1 phosphorylation. Vector or Flag-EDG-1-expressing HEK 293 cells were transfected with PDGFR-β and cultured in 10% charcoal-stripped FBS for 24 hours, metabolically labeled in phosphate free DMEM with [^{32}P]orthophosphate (70 μCi/ml) for 2.5 hours at 37 °C, then stimulated with SPP (100 nM) or PDGF (20 ng/ml). Cell lysates were prepared and immunoprecipitated with anti-Flag M2 antibody (Sigma) as described (15). Immunoprecipitates were either separated on 10% SDS-PAGE, transblotted to nitrocellulose, and autoradiographed (upper panel) or immunoblotted with anti-Flag antibody (lower panel). (**E**) β-Arrestin translocation in HEK 293 cells co-transfected with β-arrestin2-GFP, EDG-1 and empty vector for SPHK1 (green cells). Conditioned medium from β-arrestin2-RFP, EDG-1 and SPHK1 transfectants (red cells) that had been treated with 10 μM sphingosine to generate intracellular SPP (**a**) or the cells themselves (**b, c**) were added to "green" cells. β-arrestin2-GFP and β-arrestin2-RFP fluorescence was visualized using dual

excitation (488, 568 nm) and emission (515-540 nm, GFP; 590-610 nm, RFP) filter sets (10).

Fig. 4. Effect of edg-1 deletion on PDGF signaling. (A) Deletion of *edg-1* has no effect on PDGF-induced tyrosine phosphorylation of PDGFR. Wild type and EDG-1 -/- MEFs were serum starved for 24 h, and then treated without or with PDGF-BB (20 ng/ml) for 5 min. Equal amounts of cell lysate proteins were analyzed by Western blotting with anti-phosphotyrosine. Blots were then stripped and re-probed with polyclonal anti-PDGFR (Upstate Biotechnology, Inc.). **(B)** EDG-1 deletion or inhibition of sphingosine kinase diminishes PDGF-mediated Rac activation. Wild-type and EDG-1 -/- MEFs were treated with PDGF-BB (50 ng/ml) for the indicated times in the absence or presence of pretreatment with DMS (20 μM) for 20 min as indicated. Cell lysates were incubated with immobilized PAK-1 binding domain (Upstate Biotechnology) and associated GTP-Rac was determined by Western blotting using a specific Rac antibody or used without affinity immunoprecipitation to determine total Rac levels as shown below (4). **(C)** Activation of EDG-1 by PDGF. Scheme for intracellular communication between tyrosine kinase growth factor receptor (PDGFR) and GPCR (EDG-1) signaling pathways.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HEK 293
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EDG-1
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10
8
6
4
2
0
*
*
None
PDGF
SPP
FBS
FN
B
MEF
WT
-/-
Chemotaxis (10² Cells /Field)
8
7
6
5
4
3
2
1
0
*
*
None
PDGF
SPP
FBS
FN
C
ASMC
Control
Sense
Antisense
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2
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6
4
2
*
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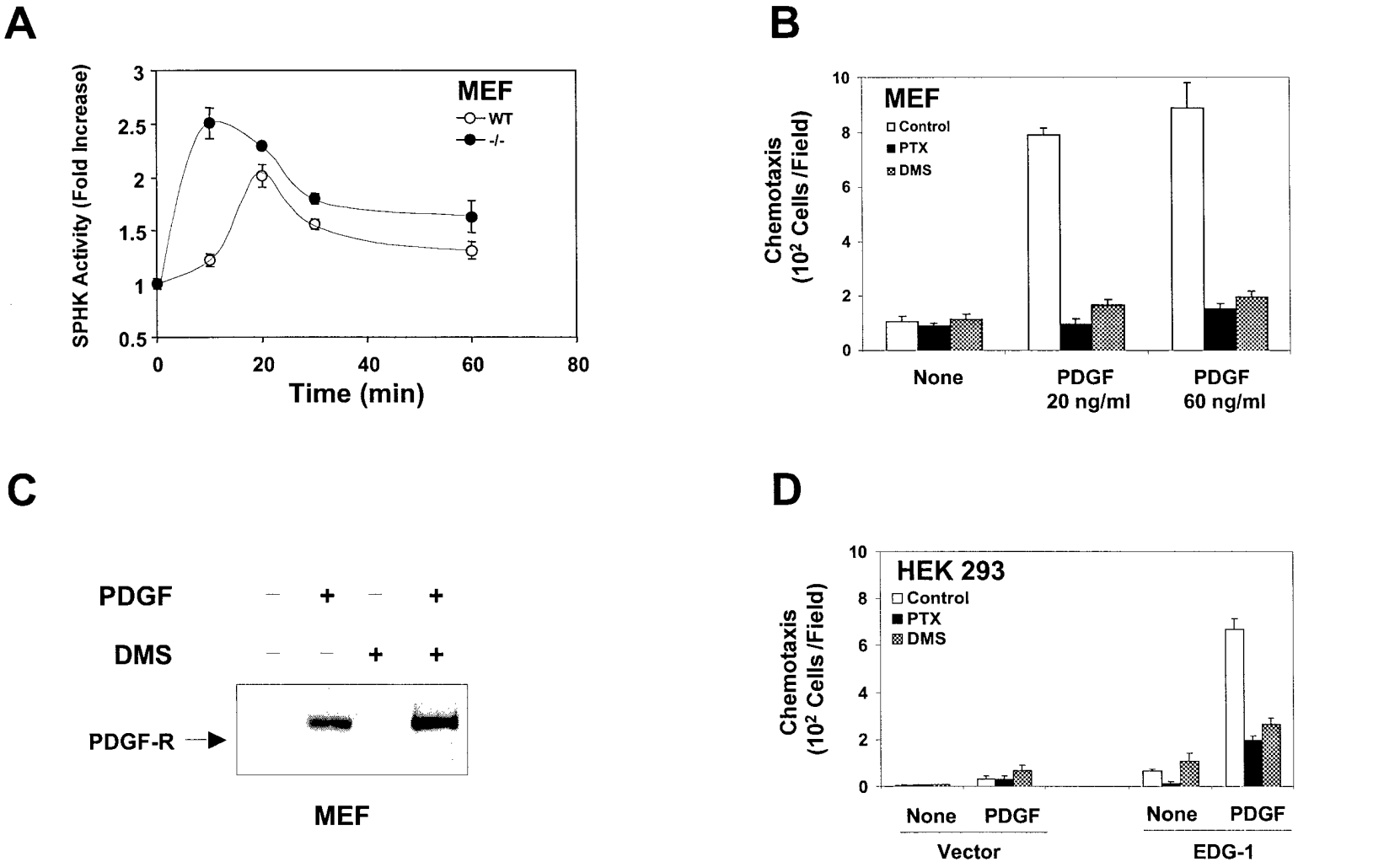
SPP
PDGF
FBS

Figure 1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MEF
WT
-/-
Time (min)
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MEF
Control
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DMS
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PDGF 20 ng/ml
PDGF 60 ng/ml
C
PDGF
DMS
PDGF-R
MEF
D
Chemotaxis (10² Cells /Field)
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PTX
DMS
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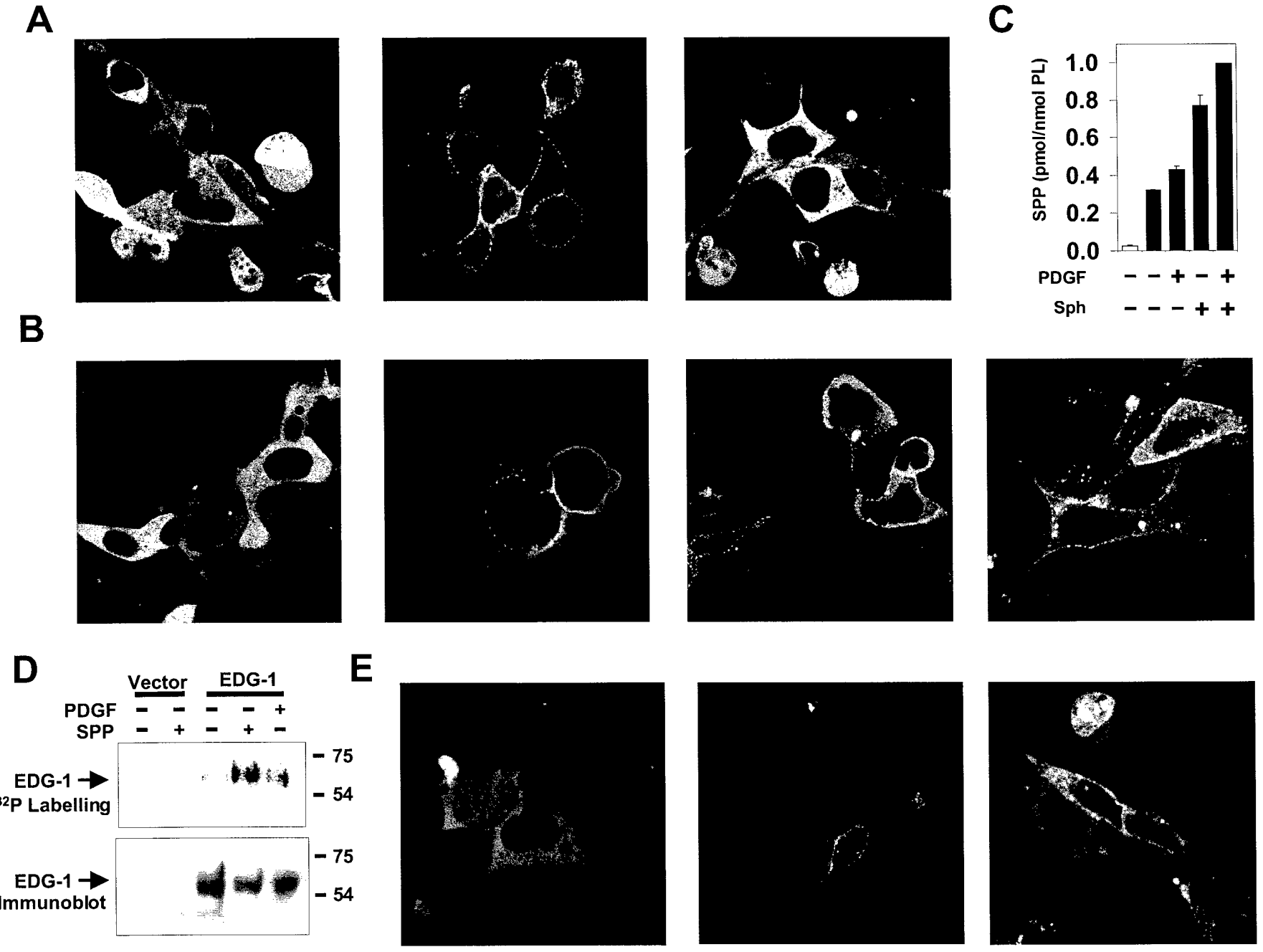
PDGF
Vector
EDG-1

Figure 2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B
C
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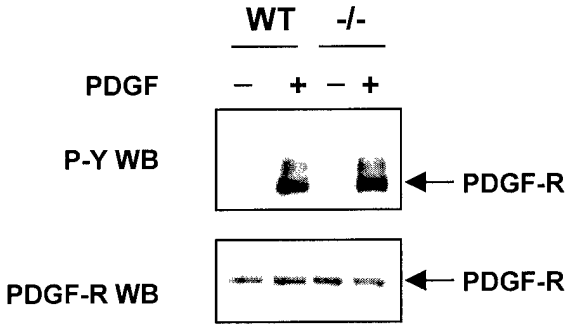
0.6
0.4
0.2
0.0
PDGF
Sph
D
Vector
EDG-1
PDGF
SPP
EDG-1
32P Labelling
EDG-1
Immunoblot
75
54
75
54
E

Figure 3

A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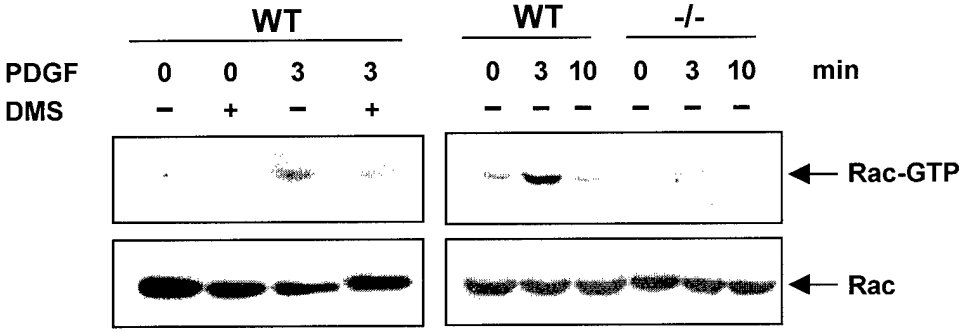
-/-
PDGF
– + – +
P-Y WB
PDGF-R
PDGF-R WB
PDGF-R

B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WT
-/-
PDGF 0 0 3 3 0 3 10 0 3 10 min
DMS – + – + – – – – – –
Rac-GTP
Rac

C

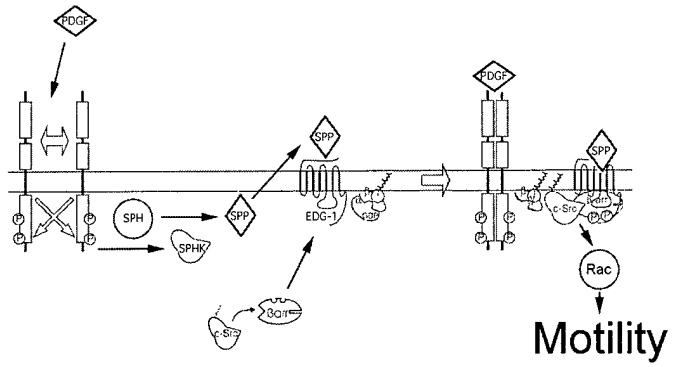
PDGF
SPH
SPHK
SPP
EDG-1
c-Src
Rac
Motility

Figure 4