

Rapid Communication

Induction of Endothelial Cell Chemotaxis by Sphingosine 1-Phosphate and Stabilization of Endothelial Monolayer Barrier Function by Lysophosphatidic Acid, Potential Mediators of Hematopoietic Angiogenesis

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ABSTRACT

Angiogenesis, the formation of new blood vessels, is an important component of restoration of hematopoiesis after BMT, but the mediators involved in hematopoietic angiogenesis have not been identified. We examined the influence of the lipid growth factors, phosphatidic acid (PA), lysophosphatidic acid (LPA), and sphingosine 1-phosphate (S1P), on several angiogenic properties of endothelial cells, including migration and stabilization of vascular barrier integrity. In a previous study, PA was found to disrupt the permeability of established endothelial monolayers, an early event in the angiogenic response that liberates cells for subsequent mobilization. In the present study, both PA and LPA weakly induced the chemotactic migration of endothelial cells from an established monolayer. The chemotactic response induced by PA and LPA was similar in intensity to that observed with optimal levels of the known protein endothelial cell chemoattractants, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). A markedly greater chemotactic response was effected by nanomolar concentrations of S1P, indicating that this platelet-derived factor plays an important role in a key aspect of angiogenesis, chemotactic migration of endothelial cells. The chemotactic response to S1P was completely inhibited by preincubation of endothelial cells with antisense oligonucleotides to the high-affinity S1P receptor, Edg-1. In addition, chemotaxis of endothelial cells to S1P was inhibited by preincubation of cells with specific inhibitors of tryrosine kinases, but inhibitors of phosphatidylinositol 3' kinase had little effect. Finally, LPA effectively stabilized endothelial monolayer barrier function, a late event in angiogenesis. Thus, the phospholipid growth factors, PA, S1P, and LPA, display divergent and potent effects on angiogenic properties of endothelial cells and giogenic differentiation of endothelial cells potentially act in tandem to effectively induce neovascularization. These mediators may thus exert important roles in restoration of hematopoiesis, as they facilitate blood vessel formation at sites of transplanted stem cells, allowing the progeny of engrafted progenitors to move from marrow sinusoids to the peripheral vasculature.

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INTRODUCTION

ANGIOGENESIS, THE FORMATION OF NEW BLOOD VESSELS (1,2), is a critically important component of hematopoiesis. After BMT, angiogenesis is required to supply necessary nutrients and oxygen to implanted progenitor cells. Subsequently, new blood vessels are necessary to transport progeny of proliferating and differentiating progenitors out of the marrow sinusoids and into the peripheral circulation. The mediators of hematopoietic angiogenesis have not been identified, but the phospholipid growth factors released from stimulated platelets (3–6), including phosphatidic acid (PA), lysophosphatidic acid (LPA), and sphingosine 1-phosphate (S1P), are attractive candidates. Recently, the endothelial differentiation gene (Edg-1) receptor, originally cloned from RNA induced in endothelial cells undergoing angiogenic differentiation (7), was demonstrated to be a high-affinity receptor for S1P (8–10), indicating an important role for this phospholipid in the angiogenic response. Other receptors of the Edg family have been demonstrated to ligate and respond to other phospholipid growth factors, including Edg-2 and Edg-4, which respond to both LPA and PA (for reviews, see references 11 and 12). Thus, although the phospholipid growth factors that ligate the Edg receptor family potentially exert important effects in angiogenesis, the effects of their ligands on endothelial cell angiogenic responses have not been thoroughly assessed.

Angiogenesis is a multifaceted process involving permeability and liberation of cells from established monolayers, chemotactic migration, proliferation, and stabilization of newly formed vessels. LPA has been demonstrated to be a remarkably effective endothelial cell mitogen (13), and S1P has been shown to induce calcium mobilization (14), adhesion molecule expression (15), and suppression of apoptosis in endothelial cells (16). The effect of S1P on cell migration has been studied previously, but to date, only inhibitory effects have been reported (17–20). Recently, we demonstrated that PA effectively induced permeability of endothelial monolayers (21), a prerequisite of new vessel formation. An analogous situation has been observed with the protein growth factor, vascular endothelial growth factor (VEGF) (22), which induces monolayer permeability, allowing liberated endothelial cells to migrate, proliferate, and differentiate to form a new vascular bed. Following these events, factors must operate to stabilize newly formed vessels, providing an intact vascular barrier to efficiently bring nutrients to and products from the newly vascularized tissue. The present study was undertaken, therefore, to examine angiogenic responses of the lipid growth factors on endothelial cells. The results demonstrate that S1P is a potent endothelial cell chemoattractant that exerts its effect by activating a novel signaling pathway initiated

by ligation of Edg-1. S1P may work in concert with PA and LPA and other growth factors to initiate and potentiate hematopoietic angiogenesis, optimizing new blood vessel formation and release of hematopoietic progeny after restoration of hematopoiesis following BMT and reversal of marrow hypoplasia.

MATERIALS AND METHODS

Phospholipids (including synthetic S1P, dioleoyl PA, and C-1 oleoyl LPA), bovine thrombin, and other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. Metabolic inhibitors, including genistein, herbimycin A, wortmannin, LY-294002, and PP2, were from Calbiochem (LaJolla, CA) and were dissolved in DMSO at a concentration of 1 mM. Modified Boyden chemotaxis chambers (6.5 mm diameter, 8 μ m pore size) were obtained from Costar, Inc. (Cambridge, MA). Recombinant human VEGF and basic fibroblast growth factor (bFGF) were from R&D Systems, Inc. (Minneapolis, MN), and were resuspended in an aqueous solution of fatty acid-free HSA at a concentration of 100 μ g/ml. Bovine pulmonary artery endothelial cells (BPAEC) were obtained from Cell Systems Inc. (Kirkland, WA) and propagated in DMEM supplemented with endothelial growth supplement and nonessential amino acids, as previously described (23). Rat tail collagen was obtained from Boehringer Mannheim (Indianapolis, IN) and dried overnight onto chemotaxis filters (2.0 μ g/filter), unless otherwise indicated.

Migration assays

BPAEC between passages 5 and 15 were dislodged after brief trypsinization and dispersed into homogeneous single cell suspensions that were washed extensively and resuspended in DMEM at a concentration of 10^6 cells/ml. To assess migration from established monolayers, cells (10^5) were dispersed onto collagen-coated chemotaxis filters within Boyden chamber inserts and allowed to adhere for 2 h at 37°C, after which they were challenged by the addition of 300 μ l of a chemoattractant solution to the lower compartments. Migration was allowed to proceed for 2 h at 37°C. Cells remaining attached to the upper surface of the filters were carefully removed with a cotton swab. Cells that had migrated to the lower surface were fixed with formaldehyde, stained with hematoxylin, and enumerated by microscopic examination. The average number of migrating cells per field was assessed by counting at least four random fields per filter at 200 \times magnification. Data points indicate the mean (\pm SD) obtained from three separate chambers within one representative experiment. Although the relative activity of different samples was consistent among experiments,

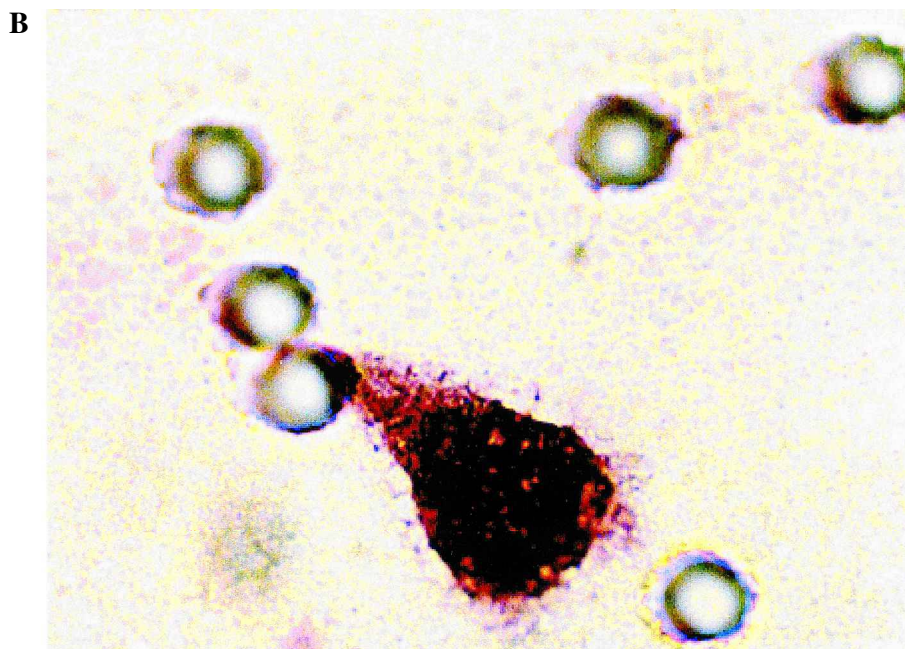
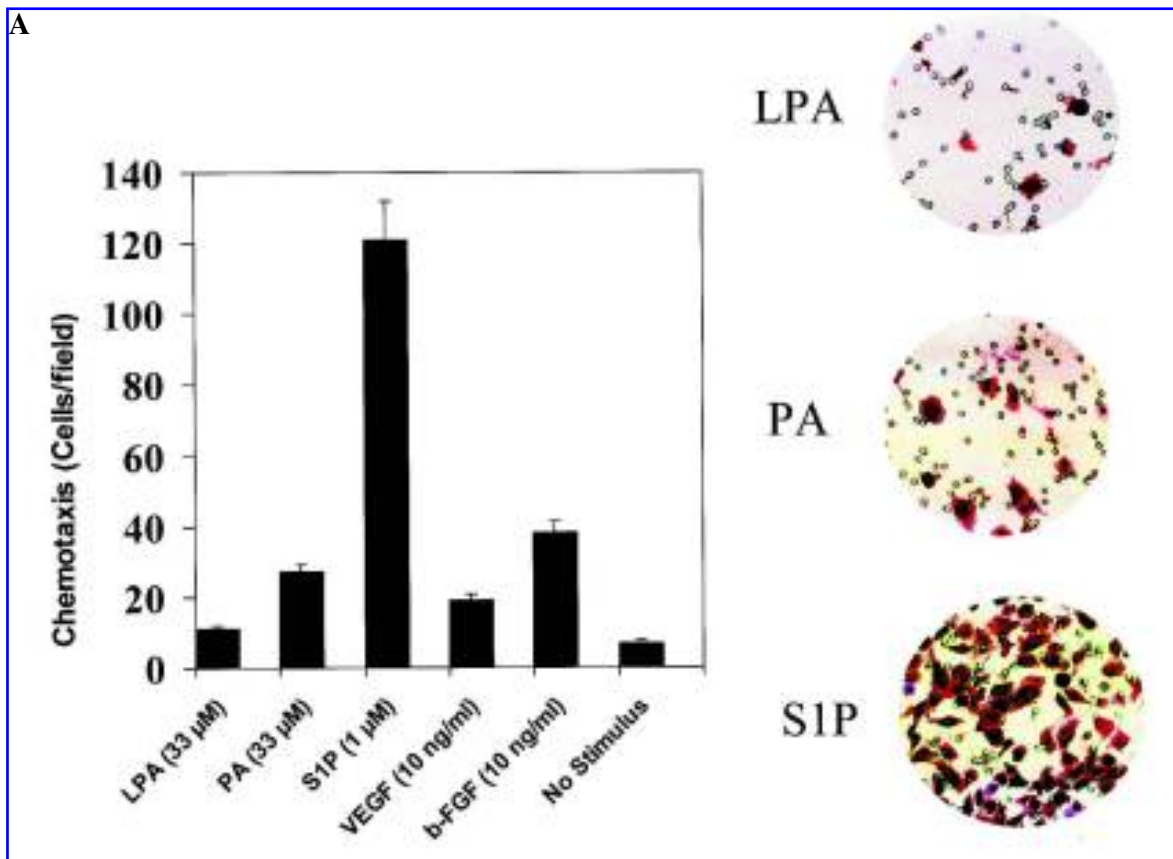


FIG. 1. (A). Chemotactic effect of PA, LPA, and S1P for BPAEC. Results obtained with the protein endothelial cell chemoattractants are shown for comparison. Results depicted for PA, LPA, and S1P were the optimal obtained from assays conducted over a wide concentration range (10 nM–50 μ M). S1P exerted substantial responses at levels as low as 10 nM and optimal responses at levels of approximately 1 μ M in this serum-free system. Photomicrographs of the lower surface of chemotaxis filters stained with hematoxylin and eosin after migration to the indicated phospholipid are shown at right (200 \times). **(B)** An individual endothelial cell as it emerges through an 8- μ m pore on the underside of the chemotaxis filter in response to 100 nM S1P. Nuclear material is evident at the anterior of the cell as it enters its new environment, with cytoplasmic streaming leading back to the transversed micropore.

the magnitude of optimal responses expressed as the average number of cells per field varied, partly due to use of cells in different passages.

Treatment of endothelial cells with Edg-1 antisense oligonucleotides

Phosphorothioate sense and antisense oligonucleotides were synthesized by Ana-Gen Technologies Inc. (Palo Alto, CA) based on the published human Edg-1 sequence (7). The Edg-1 antisense oligonucleotide (GACGCTGTGGGCCCCAT) was complementary to the first 18 bases of the coding region beginning at the initiating ATG, and the Edg-1 sense oligonucleotide, used for comparison, was complementary to this sequence (ATGGGGCCACCAGCGTC).

BPAEC growth to 80%–90% confluence in 25-cm² flasks were treated for 4 h with 2 μ g (approximately 0.36 μ M, final concentration) of oligonucleotides complexed with 25 μ l Lipofectamine (GIBCO-BRL, Gaithersburg, MD) in 1 ml OptiMEM (GIBCO-BRL) in accordance with the manufacturer's instructions. An equal volume of complete endothelial growth medium containing 40% FBS was then added directly to flasks, and the cells were incubated overnight in the transfection mix. The medium was replaced the next day, and the cells were incubated for an additional 24 h before they were harvested for chemotaxis assays.

Endothelial monolayer barrier integrity

Endothelial monolayer barrier integrity was measured by determination of electrical resistance of BPAEC monolayers using an electrical cell impedance sensor (ECIS) (Applied Biophysics, Inc., Troy, NY) as previously described (24,25). In this system, endothelial cells were cultured on a small gold electrode (10⁻⁴ cm²) in DMEM supplemented with 20% (v/v) colostrum-free bovine serum, antibiotics, and growth factors as previously described. Before each experiment, medium with serum was replaced with the same medium without serum, which was used as the electrolyte. In this assay, cells act as insulating particles, and the total resistance across the monolayers is composed of the resistance between the ventral cell surface and the electrode and the resistance between cells. A 4000-Hz AC signal with 1 V amplitude was applied to the endothelial monolayers through a 1 M Ω resistor, creating an approximate constant current source (1 μ A). The lockin amplifier attached to the electrodes detects changes in both magnitude and phase of the voltage appearing across the cellular monolayer and was controlled by a computer that was used to both initiate the experiments and process the data. Electrical resistance increased immediately after cells attached and covered the electrodes, and the resistance

achieved a steady state when the monolayers became confluent. Thus, experiments were initiated after the electrical resistance achieved a steady state. Resistance data were normalized to the initial voltage and plotted as normalized resistance.

RESULTS AND DISCUSSION

Chemotactic effect of S1P and related lipids

Figure 1 shows the influence of S1P, LPA, and PA on endothelial cell chemotaxis. Although each of these phospholipids effectively induced chemotaxis under serum-free conditions, the most prominent response by far was observed with S1P, at an optimal concentration of 1 μ M. Under these conditions, S1P was a much stronger endothelial cell chemoattractant than optimal concentrations of either bFGF or VEGF, which effected responses approximately equal in intensity to those observed with optimal levels of PA and LPA, respectively. Thus, S1P is a potent endothelial cell chemoattractant that may play an important role in inducing endothelial cell migration to sites of neovascularization.

Involvement of Edg-1 in S1P-induced chemotaxis of endothelial cells

Experiments were undertaken to establish the endothelial cell receptor that drives the chemotactic response initiated by S1P and the signaling pathway activated by receptor binding. As shown in Figure 2, pretreatment of endothelial cells with antisense oligonucleotides to the high-affinity S1P receptor, Edg-1, completely attenuated the chemotactic response to S1P. In contrast, sense oligonucleotides had no effect. Thus, S1P induces endothelial cell chemotaxis by ligating the Edg-1 receptor.

We next examined postreceptor events that led to the migratory response induced by S1P. As shown in Figure 3, chemotaxis of endothelial cells to S1P was strongly inhibited by several tyrosine kinase inhibitors, including genistein (26), herbimycin A (27), and the specifically acting src kinase inhibitor, PP2 (28). However, inhibitors of phosphatidylinositol 3' kinase, wortmannin, and LY-294002 had little effect on migration when used at levels effective in blunting chemotaxis and calcium mobilization of neutrophils to PA and PA-enriched plasma membranes (29,30). Interestingly, chemotaxis of neutrophils to PA is also inhibited by the tyrosine kinase inhibitors, genistein and herbimycin A (31). Taken together, these results indicate that S1P activates endothelial cell migration by inducing a novel signaling pathway initiated by ligation of the Edg-1 receptor.

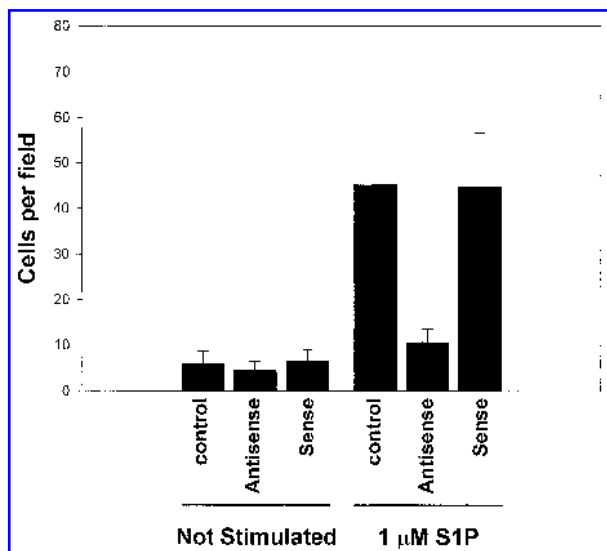


FIG. 2. Effect of Edg-1 phosphorothioate sense and antisense oligonucleotides on endothelial cell chemotaxis to S1P. Cells grown to 80% confluence were treated for 4 h with sense or antisense oligonucleotides, allowed to recover for 24 h, and harvested for chemotaxis assays. Results depict mean \pm SD of three determinations.

Effect of phospholipid growth factors on endothelial monolayer stability

In a previous report, we documented the effect of PA on endothelial monolayer permeability (21). PA was found to disrupt permeability of established endothelial

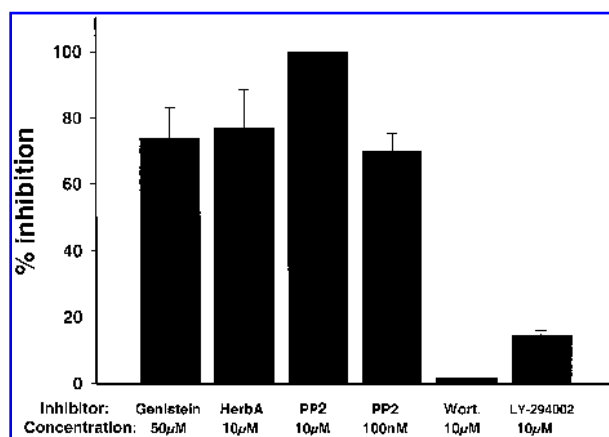


FIG. 3. Effect of kinase inhibitors on S1P-induced endothelial cell chemotaxis. The tyrosine kinase inhibitors genistein and herbimycin A (HerbA) exerted potent inhibitory effects, as did the specifically acting src inhibitor PP2. However, little inhibition was noted with either wortmannin (Wort.) or LY-294002, effective inhibitors of phosphatidylinositol 3' kinase that block PA-induced chemotaxis of neutrophilic leukocytes (29). Results shown are from one experiment that was repeated twice for confirmation.

monolayers, a result that may be relevant to both the pathology of pulmonary edema and the process of angiogenesis. VEGF, a potent angiogenic agent, effectively disrupts endothelial monolayer permeability, and this process is thought to play an important, if not essential, role in the genesis of angiogenesis (32). Increased permeability may facilitate angiogenesis by ef-

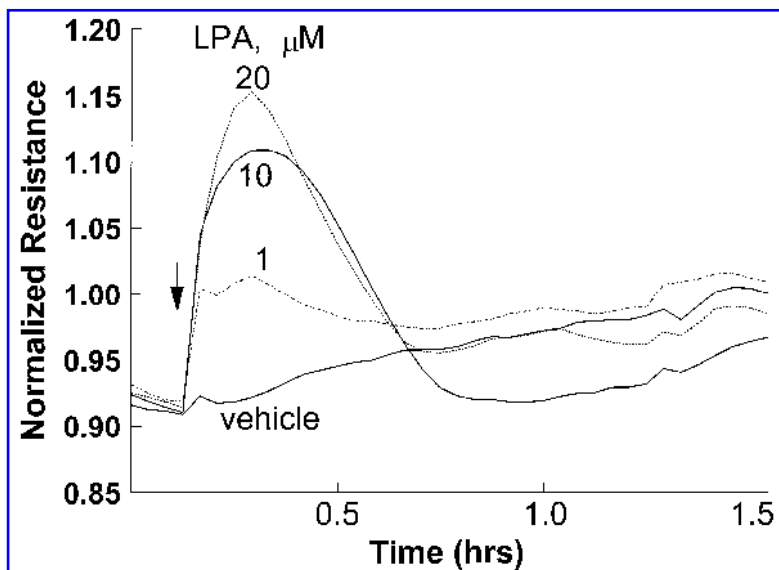


FIG. 4. Stabilization of endothelial cell monolayers by lysophosphatidic acid (LPA). Endothelial cell monolayer resistance was monitored for 1.5 h. One hour before the experiment, cells were rinsed with medium to remove serum, and at the time indicated by the arrow, cells were treated with either vehicle (4 μ g/ml BSA in PBS) or different doses of LPA (1–20 μ M). Shown are results from a representative experiment ($n = 3$).

fecting extravasation of essential plasma proteins or by facilitating endothelial cell proliferation and migration as a consequence of monolayer destabilization (32). Moreover, vessels formed in response to VEGF are leaky and not functionally optimal (32,33), suggesting other factors work in tandem with VEGF (and perhaps other angiogenic agents) to stabilize endothelial monolayer barrier function after neovascularization, thereby allowing new vessels to function optimally. Therefore, we assessed the influence of LPA on stabilization of endothelial monolayer barrier function. As shown in Figure 4, LPA effectively enhanced barrier function of newly formed endothelial cell monolayers, as assessed by assays of electrical resistance effected by cellular monolayers. Thus, the phospholipid growth factors, PA, S1P, and LPA, may operate in tandem to first liberate endothelial cells from established vessels, induce their directional migration, and finally stabilize newly formed vessels.

Conclusions

The experiments in this report demonstrate that S1P is an extremely effective endothelial cell chemoattractant that exerts its effect by ligating Edg-1, a high-affinity G-protein-coupled receptor. After ligation, migration is induced by activation of a signaling pathway that involves activation of src-tyrosine kinases but not phosphatidylinositol 3' kinase. S1P may exert important effects on angiogenesis in combination with other phospholipid growth factors, including PA and LPA, which may affect endothelial cells by binding other members of the Edg receptor family. In addition, S1P, through its potent endothelial cell chemotactic potential, may play an important role in angiogenic responses induced by various protein angiogenic factors, including VEGF and FGF.

Although S1P and related lipids are known to be released by stimulated platelets, these lipids are rapidly taken up and hydrolyzed by cells in blood and other tissues (34). Indeed, because of the hydrophobic nature of S1P, extracellular export of the phospholipid has been difficult to detect (34). Once released, S1P is rapidly hydrolyzed by vigorous ecto-phosphatases present on the outer surface of neutrophils and other cells in blood (35,36). Although certain biologic activities of serum have been traced to the presence of LPA (37), to our knowledge, no one has directly traced the biologic activity of serum or other tissue extracts to the presence of S1P. At present, therefore, there is no information pertaining to the contribution of cell-released S1P to the angiogenic properties of serum and other tissue extracts. Although cells respond to exogenously applied S1P with a multitude of effects (4,6,8,10,11,16,20,36), the physiologic significance of endogenously released S1P has not been defined. Recently, we demonstrated

that endogenously generated and released S1P is capable of inducing several angiogenic responses of endothelial cells, including chemotaxis, angiogenic differentiation, and blood vessel formation in vivo (D. English et al., unpublished observations). Experiments are underway to further define the role of endogenously generated S1P and other lipid mediators in the angiogenic response and to determine the physiologic role of platelet-released S1P in wound healing and hematopoietic angiogenesis.

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