Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization

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Vascular stabilization, a process by which nascent vessels are invested with mural cells, is important in angiogenesis. Here we describe the molecular basis of vascular stabilization regulated by sphingosine 1-phosphate (S1P), a platelet-derived lipid mediator. S1P1 receptor-dependent cell-surface trafficking and activation of the cell–cell adhesion molecule N-cadherin is essential for interactions between endothelial and mural cells. Endothelial cell S1P1/Gi/Rac pathway induces microtubule polymerization, resulting in trafficking of N-cadherin to polarized plasma membrane domains. S1P treatment modulated the phosphorylation of N-cadherin as well as p120-catenin and induced the formation of cadherin/catenin/actin complexes containing novel regulatory and trafficking factors. The net result of endothelial cell S1P1 receptor activation is the proper trafficking and strengthening of N-cadherin-dependent cell–cell adhesion with mural cells. Perturbation of N-cadherin expression with small interfering RNA profoundly attenuated vascular stabilization in vitro and in vivo. S1P-induced trafficking and activation of N-cadherin provides a novel mechanism for the stabilization of nascent blood vessels by mural cells and may be exploited to control angiogenesis and vascular diseases.

Keywords: sphingosine 1-phosphate; cadherin; angiogenesis; vascular stabilization; endothelial cells; pericytes

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Vascular stabilization, which is defined as the investment of mural cells (pericytes and vascular smooth muscle cells [VSMC]) to the endothelial cell [EC] layer of nascent vessels, is critical for vascular development, angiogenesis, and the maintenance of the established vasculature [Hirschi and D’Amore 1997; Jain 2003]. Mutation of vascular regulatory genes [growth factors, receptors, signaling molecules] causes cardiovascular system failure and embryonic lethality, which are often due to abnormal mural cell investment of nascent vessels [Yancopoulos et al. 2000]. During the development of the retina, abnormal mural cell coverage of the nascent blood vessels leads to compensatory angiogenesis and dysfunctional retinal development [Risau 1997; Benjamin et al. 1998]. In the adult, vasotoxic stresses [hypoxia, hyperglycemia] induce mural cell loss from established microvessels, leading to abnormal vascular permeability, uncontrolled angiogenesis, and ultimately tissue destruction [Benjamin et al. 1998]. This is exemplified prominently in diabetic retinopathy, in which angiogenic vessels fail to stabilize properly, eventually leading to blindness [Engerman 1989; Hammes et al. 2002]. Moreover, tumor angiogenesis, a process critical for the growth of solid tumors, also requires vascular stabilization [Bergers et al. 2003]. Inhibition of vascular stabilization leads to attenuated tumor angiogenesis and reduced growth rate of tumors. Therefore, vascular stabilization [also known as maturation] is considered to be an important process in development, physiology, and pathology [Jain 2003].

Despite the importance of this phenomenon, molecular mechanisms involved are poorly understood. EC/mural cell coculture studies have identified PDGF-BB and TGF-β as important regulators of this process [Hirschi et al. 1998]. However, our understanding of vascular stabilization at the cellular and molecular levels is limited.

The S1P1 receptor [also known as EDG-1], a G-protein-coupled receptor (GPCR) for the bioactive lipid S1P, was originally cloned from vascular EC as an inducible gene [Hla and Maciag 1990; Lee et al. 1998]. Previous work has implicated this receptor as a major regulator of EC func-
Mechanism of vascular maturation

**Results**

**SIP1 receptor is required for EC–mural cell interaction**

The phenotype of the S1P1 knock-out mice suggests that EC interaction with mural cells may be deficient [Liu et al. 2000]. We therefore tested whether S1P would modulate EC–mural cell interactions in an in vitro coculture system [Hirschi et al. 1998]. First, we isolated and established murine embryonic EC (MEEC) lines from S1p1+/+ mouse embryos as described (Fusco et al. 1998; Balconi et al. 2000). We therefore tested whether S1P would modulate EC–mural cell interactions in an in vitro coculture system [Hirschi et al. 1998]. First, we isolated and established murine embryonic EC (MEEC) lines from S1p1+/+ mouse embryos as described (Fusco et al. 1998; Balconi et al. 2000). When MEEC were cocultured with fluorescently labeled 10T1/2 cells (a mesenchymal cell line with pericyte properties), S1P induced (three- to fourfold after 0.5 h) 10T1/2 cell binding to wild-type and heterozygous but not with S1p1−/− MEEC (Fig. 1a,c). Fluorescence micrographs show that S1P treatment induced 10T1/2 cell interaction with wild-type MEEC via podlike processes [Fig. 1a,b]. Morphologically, this resembles pericytes interacting with EC in postcapillary venules [Allt and Lawrenson 2001]. This effect was specific, as related lipids that are not agonists for the S1P1 receptor failed to induce this interaction [Fig. 1d]. Cell–cell interaction between S1p1−/− MEEC and 10T1/2 cells was restored upon expression of the S1P1 receptor in S1p1−/− MEEC (Fig. 1e). These data suggest that S1P interaction with the S1P1 receptor on endothelial cells is critical for interaction with 10T1/2 cells.

We also tested the effect of other regulators of vascular maturation such as PDGF-BB, angiopoietins 1 and 2, and VEGF. Only S1P was effective in this cell–cell interaction assay, suggesting that it acts in a specific manner to induce cell–cell interaction between EC and mural cells (Supplementary Fig. 1a). In addition, several additional lines of VSMC were tested to rule out the possibility that this effect is a peculiarity of the 10T1/2 cells. Two different VSMCs with distinctive phenotypes, specifically that from adult aorta and embryonic aorta, responded to S1P-induced adhesion to wild-type but not to S1p1−/− MEEC [Supplementary Fig. 1b]. The adult VSMC and 10T1/2 cells do not express detectable levels of the S1P1 receptor whereas the embryonic VSMC express this receptor constitutively [Kluk and Hla 2001]. Likewise, human umbilical vein endothelial cells (HUVEC) also behaved similarly to the wild-type MEEC. These data suggest that S1P interaction with S1P1 receptor on EC regulates EC/mural cell interaction in trans, regardless of the status of this receptor on mural cells and support that the in vitro cell–cell interaction system faithfully recapitulates an aspect of vascular maturation.
N-cadherin is necessary for S1P₁ receptor-induced EC–mural cell interaction

N-cadherin, which is expressed in both EC and mural cells, was proposed to be important for the interactions between these two cell types [Dejana 2004]. Specifically, injection of anti-N-cadherin antibodies into the ventricles of chick embryonic brain induced vascular defects [Gerhardt et al. 2000]. We utilized the small interfering RNA (siRNA) approach to inhibit N-cadherin expression in EC to determine the requirement for this adhesion molecule in S1P-induced cell–cell interaction. As shown in Figure 2a, the siRNA for N-cadherin but not the scrambled counterpart potently blocked its expression in EC. Treatment of EC with the N-cadherin siRNA but not the scrambled siRNA nor the irrelevant E-cadherin siRNA inhibited the interaction between EC and 10T1/2 cells [Fig. 2b]. Additional experiments were done to confirm the specificity of the siRNA for N-cadherin. First, chicken N-cadherin or N-cadherin-GFP, which have distinct sequences at the siRNA-binding site, was not silenced [Supplementary Fig. 2]. When chicken N-cadherin-GFP was transfected into endothelial cells, its expression and function [as assessed by N-cadherin bead binding; see below] was not suppressed by the siRNA for N-cadherin. In contrast, murine N-cadherin-GFP transfection was fully suppressed in a sequence-specific manner [Supplementary Fig. 3]. S1P-induced EC and 10T1/2 adhesion was strongly inhibited by chelation of extracellular calcium but not by the inhibitors of integrins such as the RGD peptide or echistatin [Fig. 2b]. This confirms the requirement for calcium-dependent cell–cell adhesion.

**Mechanisms involved in S1P₁ regulation of N-cadherin localization and function were further investigated. We used latex beads coated with N-cadherin ectodomain-Fc chimera (NcadFc) to measure N-cadherin-dependent adhesion in EC [Lambert et al. 2000]. Binding of NcadFc-coated beads to EC increased markedly upon S1P addition [Fig. 3a,b]. In contrast, S1P treatment did not alter the binding of beads coated with CD31/PECAM antibody or a control mouse IgG to EC. These data indicate that N-cadherin is translocated to the cell surface upon activation of the S1P₁ receptor and suggest that the cell-surface-localized N-cadherin is active in binding to other N-cadherin molecules in trans.

To further demonstrate the cell-surface trafficking of N-cadherin upon S1P treatment, a cell-surface biotinylation experiment was conducted, followed by avidin affinity selection and immunoblot analysis. Significantly more N-cadherin [but not CD31 or VE-cadherin] was biotinylated on the cell surface after S1P treatment of EC [Fig. 3c], suggesting that S1P induces the trafficking of endogenous N-cadherin to the EC surface.

Cadherin-dependent junction formation was shown to be regulated by RhoGTPases [Dejana 2004]. In HUVEC, we showed that S1P treatment induced the activity of both RhoA and Rac1 GTPases [Paik et al. 2001]. Similarly, in wild-type MEEC, RhoA and Rac1 were rapidly activated after S1P addition. S1p₁−/− MEEC, however, exhibited only RhoA activation [Supplementary Fig. 4], suggesting that S1P₁ receptor is important in the activation of the Rac1 GTPase.

We further investigated the role of RhoGTPases in N-cadherin-dependent adhesion. S1P-induced NcadFc bead binding to EC was potently inhibited by dominant negative Rac [N17Rac] but not by dominant negative Rho [N19Rho] [Fig. 3d]. This finding was confirmed by down-regulating Rac1 expression. siRNA for Rac1 potentely silenced Rac1 protein expression [Supplementary Fig. 5]. Interestingly, diminution in Rac1 levels led to the inhibi-
bition of S1P-induced membrane ruffles and increased stress fiber formation (Supplementary Fig. 6). Concomitantly, S1P-induced NcadFc-bead binding to the cell surface was inhibited (Supplementary Fig. 7). These data indicate that Rac activation by S1P1 is essential. Moreover, time lapse microscopic monitoring of N-cadherin-GFP showed that N-cadherin-GFP was dynamically translocated to plasma membrane domains in a polarized manner. This was potently inhibited by N17Rac but not by N19Rho (Supplementary Movies 3–6).

N-cadherin trafficking to the plasma membrane was further investigated in EC treated or not with S1P using confocal immunofluorescence imaging of fixed cells. As shown in Figure 4b, endogenously expressed N-cadherin was dramatically redistributed into polarized microdomains on the EC plasma membrane. This localization correlated with the asymmetric nature of NcadFc-bead binding to the EC surface upon S1P stimulation (Fig. 3a, blue arrowheads), suggesting that N-cadherin on such domains is active. Optical sectioning and reconstruction of the z-axis of EC stained with N-cadherin antibody clearly showed a polarized distribution of N-cadherin on one side of the apical cell surface (Fig. 4b). This finding suggests the existence of specific domains containing N-cadherin on the EC surface.

**Figure 3.** S1P induces Rac1-dependent binding of NcadFc beads to EC. (a) Binding of NcadFc-beads to HUVEC surface in the absence or presence of S1P stimulation. Plasma membranes were labeled in red [DiI-C18] and N-cadherin and NcadFc-beads were stained in green [NCD]. Note that blue arrows indicate polarized binding of NcadFc-beads to EC surface. Pink arrows point to dorsal ruffles that are devoid of NcadFc-bead binding. Bar, 30 µm. (b) Beads coated with irrelevant mouse IgG [IgG], CD31-IgG, or NcadFc were used in the binding assay with S1P-treated HUVEC. The number of beads bound to the cell surface was quantified as described (**, $P < 0.001$). (c) HUVEC were treated or not with S1P, and cell-surface proteins (N-cad, CD31, VE-cad) were biotinylated and recovered by affinity isolation with Streptavidin-Sepharose. Immunoblot analysis with specific antibodies ([NB] nonbiotinylated; [WCL] whole-cell lysate) is shown. (d) Binding of NcadFc-beads to S1P-treated HUVEC expressing N17Rac or N19Rho or vector (pTet) was quantified as above (**, $P < 0.001$).

**Figure 4.** S1P induces polarized distribution of N-cadherin on the apical endothelial cell surface. HUVEC were immunostained for endogenous N-cadherin, and optical sections of z-axis of cells were reconstructed as described. (a–d) XY view and z-stack view [boxes below] of pointed lines [blue arrowheads]. N-cadherin is shown in green, nuclei are in blue [DAPI], and plasma membrane in red [DiI-C18]. (b) Note that S1P stimulation dramatically induced polarized N-cadherin distribution. This was attenuated by nocodazole treatment [2 µM, 1 h, c] but not by cytochalasin D [1 µM, 10 min, d].

**Microtubule-dependent trafficking of N-cadherin to polarized microdomains on the endothelial cell surface**

We further investigated the mechanisms involved in S1P1-induced N-cadherin translocation to the endothelial cell surface microdomains. Recently, trafficking of N-cadherin–kinesin complex along the microtubule (MT) cytoskeleton has been reported [Mary et al. 2002; Chen et al. 2003]. Therefore, we investigated whether S1P regulated MT polymerization in EC. MT growth in EC was monitored by visualizing the incorporation of the EB1-GFP protein (a specific marker for growing tips) onto growing ends of MT [Piehl et al. 2004]. S1P-stimulated HUVEC displayed marked increases in the frequency and persistency of MT growth [Supplementary Movies 7, 8]. S1P-induced increases in MT polymerization and protrusion of MT growth to the cell periphery were strongly inhibited by the dominant negative mutant of the small GTPase Rac [N17Rac] [Supplementary Movie 9]. As expected, nocodazole treatment completely inhibited MT polymerization in EC [Supplementary Movie 10]. These observations suggest that the activation of the GTPase Rac by S1P is required for efficient MT polymerization in EC.

Immunofluorescence imaging of endogenous N-cadherin in EC shows that nocodazole treatment blocked polarized cell-surface translocation [Fig. 4c] suggesting that S1P-induced MT polymerization is necessary for N-cadherin trafficking to the cell surface. In addition N-cadherin-bead binding to the EC cell surface was completely inhibited by nocodazole in a time-dependent manner [Fig. 5a].

The involvement of the actin cytoskeleton was also investigated, as it is known that the actin cytoskeleton. We further investigated the mechanisms involved in S1P1-induced N-cadherin translocation to the endothelial cell surface microdomains. Recently, trafficking of N-cadherin–kinesin complex along the microtubule (MT) cytoskeleton has been reported [Mary et al. 2002; Chen et al. 2003]. Therefore, we investigated whether S1P regulated MT polymerization in EC. MT growth in EC was monitored by visualizing the incorporation of the EB1-GFP protein (a specific marker for growing tips) onto growing ends of MT [Piehl et al. 2004]. S1P-stimulated HUVEC displayed marked increases in the frequency and persistency of MT growth [Supplementary Movies 7, 8]. S1P-induced increases in MT polymerization and protrusion of MT growth to the cell periphery were strongly inhibited by the dominant negative mutant of the small GTPase Rac [N17Rac] [Supplementary Movie 9]. As expected, nocodazole treatment completely inhibited MT polymerization in EC [Supplementary Movie 10]. These observations suggest that the activation of the GTPase Rac by S1P is required for efficient MT polymerization in EC.

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way induces the assembly of the cortical actin network [Lee et al. 1999, Paik et al. 2001]. Depolymerization of actin filaments by cytochalasin D significantly reduced NcadFc-bead binding to the EC cell surface, suggesting the critical role of actin polymerization in supporting N-cadherin-mediated adhesion [Fig. 5a]. However, S1P-induced N-cadherin trafficking to polarized plasma membrane domains was not inhibited by cytochalasin D, even though the morphology of the EC was altered [Fig. 4d]. These data suggest that the actin cytoskeleton is not involved in the trafficking of N-cadherin after S1P treatment, even though ultimate binding of N-cadherin molecules requires the formation of F-actin.

When EC were treated with nocodazole or cytochalasin D for the duration of the assay, interaction of EC and 10T1/2 cells was significantly inhibited [Fig. 5b]. As shown in Supplementary Movie 10, acute treatment [10 min] of EC with nocodazole efficiently blocked MT polymerization. However, 50 min after S1P treatment, acute nocodazole treatment did not appreciably inhibit EC–10T1/2 interactions or NcadFc-bead binding, suggesting that once N-cadherin trafficking is set into motion and sufficient N-cadherin accumulates on the plasma membrane, inhibition of the MT cytoskeleton is unable to block the junction assembly between EC and 10T1/2 [Fig. 5a,b].

**Figure 6.** S1P regulation of N-cadherin multi-protein complex in EC. (a) MEECs were treated with S1P for various times, cell extracts were prepared and immunoprecipitated with β-catenin. Immunoprecipitates were immunoblotted with antibodies for α- and β-catenin, and N-cadherin (NCD). Total amount of N-cadherin and β-catenin were also assessed in the extracts. (b) Identification of the components of multi-protein complexes of N-cadherin in HUVEC. Large-scale immunoprecipitation of N-cadherin was conducted from S1P-treated HUVEC extracts and analyzed by SDS-PAGE and Coomassie blue staining. Specific regions of the gel were cut, and protein identities were determined by mass spectrometry. [MAP] Microtubule-associated protein, [TSP] thrombospondin, [MVP] major vault protein. (c) S1P-stimulated (1 h) HUVEC lysates were immunoprecipitated for p120 catenin [p120] or NCD and blotted for phosphotyrosine [pY] or phosphoserine/threonine [pST]. Numbers are expressed as fold increases over nonstimulated conditions after normalization with the loading control [N = 3].
sin D and nocodazole. Binding of N-cadherin with cortactin was dependent on actin cytoskeleton as it was not observed in the presence of cytochalasin D. However, coimmunoprecipitation of RACK1 with N-cadherin was observed even in the presence of cytochalasin D [data not shown]. The significance of other coprecipitated proteins, such as major vault protein (MVP), leukophycin, thrombospondin [TSP1], and the translation factor Tiar1 is not known at present. These data suggest the complexity in the regulation of N-cadherin trafficking and adhesiveness in EC by multiple protein–protein interactions.

Previously cadherin/catenin phosphorylation events were shown to modulate the strength of cadherin-dependent junctions (Gottardi and Gumbiner 2001). As shown in Figure 5c, S1P stimulation potentiates the binding of NcadFc-beads to EC even in the presence of nocodazole, suggesting there are additional activation steps induced by S1P1 in addition to the trafficking to the plasma membrane. Therefore we explored the hypothesis that S1P stimulation might alter the phosphorylation of proteins in the cadherin complex and thereby alter its adhesive- ness. Phosphorylation of the N-cadherin-binding protein p120-catenin (especially the p100 isoform) was modulated by S1P treatment of HUVEC. Specifically, tyrosine phosphorylation was enhanced whereas serine/threonine phosphorylation was attenuated [Fig. 6c]. In addition, tyrosine-phosphorylated N-cadherin was also increased by S1P treatment. However, β-catenin phosphorylation was not altered significantly [data not shown]. These data suggest that alterations in p120-catenin and N-cadherin phosphorylation are likely to contribute to the changes in S1P-induced N-cadherin adhesivity.

**Aberrant localization of N-cadherin in S1p1−/− mice**

To obtain in vivo correlates of our findings, we sought to determine if N-cadherin localization in the vascular system is altered in the S1p1−/− mouse embryo. Sections of the dorsal aorta were imaged by confocal immunofluorescence microscopy. As expected, defective mural cell coverage of the dorsal aorta was observed in S1p1−/− embryos [Liu et al. 2000]. N-cadherin is expressed in EC, mural cells, and surrounding mesenchymal cells in wild-type embryos [Fig. 7]. Aberrant N-cadherin localization was observed in S1p1−/− embryos, compared to wild-type counterparts [Fig. 7a–d]. Specifically, N-cadherin localization in the intima is shifted from the basal membrane to a more lateral pattern in EC. A significant decrease in the ratio of fluorescence intensity at EC/mural cell boundaries versus that at EC/EC boundaries was observed in S1p1−/− embryos when compared to wild-type counterparts [Fig. 7e]. This mislocalization of N-cadherin was also observed in S1p1−/− MEEC in vitro, in which the N-cadherin signal was stronger at EC/EC junctions compared to that of wild-type MEEC [Fig. 7f]. Together these data support the notion that S1P1 signaling in EC is essential to induce the formation of N-cadherin-based junctions between EC and VSMC in vivo. This model is shown schematically in Figure 7g.

**Silencing of S1P1 or N-cadherin with siRNA suppresses neovessel stabilization**

Based on the hypothesis that N-cadherin function is critical for vessel stability, we explored whether suppression of its expression blocks the formation of mature vessels. We used the siRNA technology [Elbashir et al. 2001] to determine the requirement for S1P1/N-cadherin pathway.

The role of S1P1/N-cadherin in neovessel formation and stability was tested in the rat aortic ring angiogenesis assay [Nicosia and Ottinetti 1990]. Ex vivo treatment with VEGF and S1P induced new vessel sprouts that are invested with α-smooth muscle actin positive cells [Fig. 8a–c]. These cells also expressed desmin, a marker for mural cells [data not shown]. Treatment with N-cadherin siRNA strongly suppressed the expression of N-cadherin but not the related VE-cadherin protein [Fig. 8d]. Inhibition of N-cadherin expression profusely suppressed neovessel maturation without inhibiting vessel sprouting or proliferation of cells of the vessel wall [Fig. 8a–c].
Figure 8. Inhibition of vascular sprout maturation by N-cadherin RNAi. (a) Rat aortic rings were cultured in collagen gels in the presence or absence of VEGF/S1P. Some cultures were transfected repeatedly with siRNA for N-cadherin (NCDi) or scrambled control (scr) as described. After 7 d, vascular sprouts were quantified by analysis of phase contrast images as described. Microvessel densities were quantitated by the NIH image software and presented as fold increase over that of control cultures. Data represent a representative example from an experiment that was repeated two times. (b) Vascular maturation of neovessels was assessed by immunofluorescence microscopy of sections of aortic rings in collagen gel. PECAM (red), α-smooth muscle actin (green), and an overlay is shown. Note that siRNA for N-cadherin (NCDi, 1 µM) inhibited microvessel stabilization. Bar, 100 µm. Nonspecific staining of elastin fibers was seen with both antibodies. However, immunostaining of neovessel sprouts was specific. (c) Pericyte ensheathment of microvessels was reconstructed in three dimensions with z-series of PECAM (red) and α-smooth muscle actin (green) images obtained from representative fields. Note that suppression of N-cadherin dramatically abolished pericyte coverage of microvessels. Bar, 10 µm. (d) Protein extracts from rat aorta treated with siRNA were immunoprecipitated for N-cadherin (NCD). VE-cadherin (VECD) levels were not altered in the whole-cell lysate (WCL).

Discussion

This work defines the mechanism by which S1P1 receptor regulates vascular stabilization. Isolated EC from S1P1−/− mice failed to interact with pericyte-like 10T1/2 cells even after treatment with S1P. The coculture system, which recapitulates the in vivo phenotype allowed the deconvolution of molecular mechanisms. The effect of S1P in the coculture assay was specific, as PDGF, VEGF, and angiopoietin 1 and 2 were inactive, consistent with the fact that these factors signal via a different class of receptors. Indeed, knock-out mice for these factors exhibit defects in vascular stabilization distinct from that of the S1P1−/− mice; for example, PDGF-receptor-β knock-out mice showed reduced numbers of pericytes in brain microvessels, which contrasts sharply with the S1P1−/− mice [Hellstrom et al. 1999, 2001].

In the present study, we show that S1P interaction with S1P1 receptor in EC is critical for the regulated interaction between EC and mural cells. We observed that S1P-induced EC–mural cell interaction in vitro is not influenced by the expression level of S1P1 in mural cells but is absolutely dependent on endothelial S1P1. This agrees with the data from EC-specific deletion of S1P1, which phenocopies the global S1P1 knock-out mice, whereas a vascular smooth-muscle-specific knock-out remains viable throughout development [Allende et al. 2003]. These observations strongly argue for S1P1-dependent EC signaling in trans to the mural cells during vascular stabilization.

A salient finding of this work is that N-cadherin is a critical mediator of cell–cell adhesion between EC and mural cells. N-cadherin, a widely expressed cell–cell adhesion molecule, was previously implicated in EC–mural cell interaction. [Hellstrom et al. 1999, 2001]. However, mechanisms by which N-cadherin regulate vascular stabilization are not well understood. In the present study, we show that activation of N-cadherin by the S1P1-mediated Gβ/Rac1 signaling pathway induces MT

To test the in vivo relevance of our mechanistic findings, we used the subcutaneous implants of Matrigel supplemented with growth factors in the mouse as an in vivo model [Lee et al. 1999; Ancellin et al. 2002]. We showed previously that the formation of mature neovessels in the subcutaneous implants of Matrigel plugs required S1P signaling via the S1P1 receptor. Inclusion of siRNA for S1P1, but not the scrambled counterpart in the Matrigel plug markedly reduced transcript expression in vivo in cells that migrated into the Matrigel [Fig. 9a]. As expected, neovessel formation was strongly suppressed by S1P1 siRNA [Fig. 9b, E1]. The siRNA for N-cadherin potently down-regulated the expression of the corresponding protein in vivo, as determined by immunoblot analysis of Matrigel plug extracts (Fig. 9c).

Immunohistochemical analysis showed that siRNA for N-cadherin did not suppress neovessel formation; rather it profoundly inhibited the acquisition of α-smooth muscle actin positive mural cells [Fig. 9b, NCDi]. Immunoblot analysis showed the decrease in the total amount of α-smooth muscle actin in the Matrigel plugs. Structural alterations of neovessels and apparent extravasation of blood cells were observed in N-cadherin siRNA-treated Matrigel sections. Endothelial cells of nascent vessel were disorganized, phenocopying the S1P1−/− embryonic vasculature [Fig. 9b]. These observations further confirm that S1P1 expression is critical for neovessel formation and pericyte ensheathment. Importantly, N-cadherin expression is required for vessel stabilization in vivo.

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polymerization and N-cadherin multiprotein complex trafficking to polarized plasma membrane microdomains.

Our study defines two essential steps in the formation of S1P$_1$-induced N-cadherin-based EC–mural cell junctions. The first step involves N-cadherin trafficking to the EC surface. S1P induced the activation of the small GTPase Rac, which is essential for proper trafficking of N-cadherin via the MT cytoskeleton network. We showed the MT polymerization in response to S1P by monitoring the dynamics of EB1-GFP, a MT tip-binding protein. The mechanism by which Rac1 stimulates MT polymerization is thought to involve the activation of the protein kinase p65PAK and phosphorylation of stathmin, a MT destabilizing protein [Daub et al. 2001].

Second, we found that S1P increases N-cadherin-bead binding to cells even in the presence of nocodazole, suggesting that S1P$_1$ may regulate the adhesive activity of N-cadherin. It has been proposed that anchoring of the cadherin C terminus to the actin cytoskeleton may stabilize cadherin-mediated adhesion [Watabe et al. 1994]. We show that the GPCR S1P$_1$, p120-catenin, originally identified as a Src substrate that binds to the JMD of cadherin, may influence the dimerization or clustering of cadherin [Ozawa 2003]. Interestingly, the angiogenic factor VEGF induces the decrease in serine/threonine phosphorylation but the increase in tyrosine phosphorylation of p120-catenin in EC [Esser et al. 1998; Wong et al. 2000]. We show that S1P regulates the phosphorylation of p120-catenin in a similar manner. Further, we also show that N-cadherin tyrosine phosphorylation is induced by S1P. Indeed, p120-catenin has been shown to be strongly tyrosine phosphorylated at early times of calcium-induced keratinocyte differentiation, which leads to increased interaction between p120-catenin and cadherin [Calautti et al. 1998]. Our finding that the active signaling via the S1P$_1$ GPCR is needed for N-cadherin activation/trafficking suggest a novel mechanism in the regulation of cell–cell adhesion and vascular stabilization.

A related question is how N-cadherin activation in EC by S1P$_1$ signaling activates the mural cell N-cadherin in trans. It has been previously demonstrated that when cells are attached to the substrata coated with a high density of the adhesive ectodomain of Xenopus C-cadherin, forced clustering of the ectodomain occurs, which in turn significantly strengthened adhesiveness [Yap et al. 1997a,b]. Similarly, such clustering and activation of local N-cadherin on the EC surface by S1P$_1$ may induce clustering of N-cadherin on neighboring mural cells. This possibility needs to be tested further by sensitive biophysical methods.

Most interestingly, we have discovered that asymmetric distribution of N-cadherin is induced by S1P$_1$ signaling in EC. It should be pointed out that apical localization of N-cadherin in cultured endothelial cells does not imply a luminal distribution in vivo. Our data simply imply that N-cadherin trafficking to polarized domains on the plasma membrane is a receptor-mediated event. A recent study has demonstrated that N-cadherin is one of the earliest proteins to be expressed asymmetrically during gastrulation, and inhibition of N-cadherin leads to randomized looping of the heart, suggesting the critical role of this adhesion receptor in asymmetry determination [García-Castro et al. 2000]. However, active polarization of cadherin molecules upon extracellular stimulation has not been reported. MT has been recognized as an important determinant of polarity in different cell types. For example, neuronal polarity is determined by translocation of PAR3 to distal tips of axons by KIF3A, a plus-end-directed MT motor protein [Nishimura et al. 2004]. Epithelial polarization is often accompanied by a dramatic change in MT organization. The polarized delivery of apical (luminal) membrane proteins is influenced by MT dynamics [Mays et al. 1994]. We propose that asymmetric distribution of N-cadherin may provide a proper spatial cue in vascular maturation. Indeed, many sporadic vascular malformation diseases including Klippel-Trenaunay and Sturge-Weber syndromes have
been linked to asymmetric [left–right body axis] development of symptoms, suggesting potential correlation between vascular development and regulation of asymmetry [Cohen 2001]. Indeed, those patients are shown to exhibit clear asymmetry of cerebral and cerebellar hemispheres where N-cadherin and S1P, are known to be highly expressed.

Potent inhibitory effects of N-cadherin siRNA in the Matrigel plug assay in vivo as well as the rat aortic ring assay ex vivo show that N-cadherin is a critical component of neovessel stabilization. The absence of α-smooth muscle actin positive cells around N-cadherin-depleted neovessels may provide clues how N-cadherin functions during the maturation of angiogenic sprouts. Although N-cadherin was implicated in breast cancer cell migration in vitro [Hazan et al. 2000], down-regulation of N-cadherin by RNAi did not impede migration of 10T1/2 cells or VSMC toward PDGF or serum [data not shown]. Proliferation of VSMC in the rat aortic ring was also not affected, suggesting that N-cadherin may primarily play a role in EC/mural cell interaction. It is possible that cell–cell interaction is necessary for the coordinated mural cell migration along the EC of neovessels as “longitudinal migration” proposed previously [Carmeliet et al. 1999]. Alternatively, EC/mural cell contact may initiate the expression of α-smooth muscle actin within perivascular mesenchymal cells. Indeed, activation of TGF-β by cell–cell contact in EC/10T1/2 cells was reported as a potential mechanism of mural cell differentiation [Hirschi et al. 1998]. The fate of mural cells after N-cadherin engagement warrants further investigation.

S1P is secreted abundantly by platelets in the adult [Yatomi et al. 2001]. Although the origin of S1P in the embryo is unknown, recent data suggest that vascular EC can generate S1P in the extracellular environment by the action of the sphingosine kinase ecto enzyme [Ancellin et al. 2002; Sanchez et al. 2003]. Thus, plasma-borne S1P can regulate EC/mural cell interaction, and may also be required for physiological maintenance of the vascular tree. In addition, S1P induces NO production in EC by Akt-dependent phosphorylation of the endothelial cell nitric oxide synthase enzyme [Morales-Ruiz et al. 2001]. Therefore, an important physiological function of S1P may be to promote vascular maturation and to maintain vascular homeostasis. In this context, it has been appreciated for several decades that platelet-derived molecules are important for the preservation of EC integrity, which includes proper regulation of vascular tone, permeability, and prevention of thrombosis [Gimbrone et al. 1969]. Indeed, an experimental immunomodulator FTY720, which is phosphorylated by the sphingosine kinase-2 enzyme into a S1P receptor agonist, profoundly inhibits vascular permeability in vivo [Sanchez et al. 2003]. Although previous studies have emphasized the role of S1P and its receptors in promoting EC barrier integrity, the mechanisms identified are limited to EC junction assembly [Lee et al. 1999; Garcia et al. 2001; Schaphorst et al. 2003]. These recent data, together with the mechanistic information provided in this study, demonstrate that S1P1 receptor signaling in EC is a critical modulator of vascular stabilization in developmental and physiological contexts. Modulation of this pathway to induce vascular stabilization in a regulated manner may prove useful in various pathological conditions in which aberrant EC/pericycle interactions occur, for example, in cancer, diabetic retinopathy, sepsis, and so on. In addition, this pathway could be used to coax the formation of new collateral vessels in ischemic tissues such as the heart and the limb with the aim of reestablishing blood flow.

Materials and methods

EC/mural cell adhesion assay

Mouse embryonic endothelial cells were isolated from E12.5 embryos and immortalized with polyoma middle T carrying retroviruses as described [Carmeliet et al. 1999]. For in vitro adhesion [Hirschi et al. 1998], 10T1/2 cells were prelabeled with Vybrant (Molecular Probe) cell tracker and incubated with adherent MEEC for 1 h either in the presence or absence of 100 nM S1P, and trypsinized under mild conditions (1× Hank’s balanced salt solution, 0.01% trypsin, 1 mM CaCl2) to preserve cell surface cadherin [Gavard et al. 2004]. Labeled cells were incubated with adherent MEEC for 1 h either in the presence or absence of 100 nM S1P, other lipids, inhibitors (2 mM EGTA, 100 nM Echistatin, 0.3 mM GRGDTP RGD peptide) or cytokines (10 ng/mL PDGF-BB, 50 ng/mL VEGF, 0.6 g/mL Ang1 and 2). Unbound cells were washed and adherent cells on the plates were quantified with a fluorimeter. Alternatively, fully adherent 10T1/2 cells (containing multiple fine processes as shown in Fig. 1b) were imaged and scored after fluorescence microscopy. The results were subjected to statistical analysis by Student’s t test unless specified.

Inhibition of gene expression by siRNA

Inhibition of S1P1/EDG-1 or N-cadherin expression by siRNA was performed using specific siRNA reagents as described [Elbashir et al. 2001]. The siRNAs used are mouse S1P1/EDG-1 ssense CUGACUUCAGGUGUGUCAAdTdT and antisense UGAACACCACUGAACUCAGCdTdT, N-cadherin siRNA (UGUCAAUUGGGUGUCCACACdTdT and CUGGAGAAACCACAUUGCACdTdT), E-cadherin siRNAs (CCUGGUUCAGAUCACUGAAtt and UCAGCACGCAUCGAUCGAdTdT), C-cadherin siRNAs (CAUGUUUCAGAUCACUGAAtt and UCAGCACGCAUCGAUCGAdTdT) and scrambled siRNA (CAUGUUUCAGAUCACUGAAtt and UCAGCACGCAUCGAUCGAdTdT). Inhibition of gene expression by siRNA was performed using specific siRNA reagents as described [Elbashir et al. 2001]. For the silencing of Rac1, SMARTpool Rac1 siRNA from Dharmacon Research Inc., protected, and duplexed as described [Elbashir et al. 2001]. For testing the specificity of N-cadherin siRNA, HEK293 cells transiently expressing mouse N-cadherin were transfected with 200 nM N-cadherin siRNA for 48 h and analyzed. For NcadFca binding, mouse N-cadherin-GFP [provided by Dr. Gauthier, Centre de Recherche de Biochimie Macromoleculaire, Centre National de la Recherche Scientifique Unite Pre- re de Recherche, Montpellier, France] or chicken N-cadherin-GFP [provided by Dr. Eppenberger, Institute of Cell Biology, Federal Institute of Technology (ETH), Zurich, Switzerland]
were transfected into HUVEC along with 200 nM N-cadherin siRNA.

**Matrigel plug assay**

siRNAs were mixed with Matrigel and implanted subcutaneously as described before. Some plugs contained FGF-2 to stimulate angiogenesis. One week after injection, plugs were removed and analyzed by immunohistochemistry [Lee et al. 1999]. Alternatively, protein or RNA was extracted from plugs and gene expression was examined by Western or Northern analysis [Paik et al. 2001].

**Immunoprecipitation and immunoblotting**

Cells were lysed as described [Lee et al. 1999]. One hundred micrograms of cell lysate were incubated with anti-β-catenin or N-cadherin antibodies [Santa Cruz Biotechnology, Inc.] and immune complexes were captured with precleared protein G beads. To exclude the possibility of actin filament-mediated aggregation of proteins, cells were treated with cytochalasin D [1 μM] for 10 min before lysis and cytochalasin D was also included in the lysis buffer. For the detections of either tyrosine or serine/threonine phosphorylation on cadherins and catenins, 500 μg of lysates (prepared in CHAPS buffer with 1 mM sodium orthovanadate, 1 mM of sodium fluoride, 10 mM of β-glycerophosphate) were immunoprecipitated and probed with anti-phosphotyrosine (clone 4G10, Upstate Biotechnology) or anti-phosphoserine/threonine (clone 22a, BD Pharmingen) antibodies. Cadherin antibodies and immune complexes were captured with precleared protein G beads. For surface biotinylation, cells were exposed to 2 mg/mL sulfo-NHS-biotin (Pierce) for 10 min on ice. Biotinylated proteins were pulled down with Streptavidin beads.

**Protein identification by μ-capillary liquid chromatography and tandem mass spectrometry**

Characterization of N-cadherin associated multimeric protein complexes was performed by large-scale immunoprecipitation of N-cadherin from S1P-treated HUVEC cells (1 × 10⁷ cells or 3 mg of total cell lysate). Briefly, cells were lysed [1% Triton X-100, 1% CHAPS-containing lysis buffer], immunoprecipitated with 3 μg N-cadherin antibody, and immunoprecipitated proteins were then resolved by SDS-PAGE and visualized by Coomassie blue staining. Protein bands were then excised, dehydrated with acetonitrile, and in-gel digested with sequencing-grade Trypsin. Peptides from each of the protein bands were then extracted with a buffer containing 5% formic acid and 50% acetonitrile, dried, and analyzed by a μ-capillary LC-MS/MS procedure using a Finnigan LCQ-DECA ion-trap tandem mass spectrometer. Uninterpreted MS/MS spectra were compared against a nonredundant human protein database using the SEQUEST algorithm [Sadygov et al. 2002]. Protein coverage was analyzed by the INTERACT program and probabilities of the SEQUEST algorithm (Sadygov et al. 2002). Protein coverage was against a nonredundant human protein database using the procedure using a Finnigan LCQ-DECA ion-trap tandem mass spectrometry.

**Immunofluorescence and imaging**

Mouse embryos [E12.5] were fixed in 4% formaldehyde, soaked in 0.3 M sucrose overnight, and cryostat sectioned. Five-micron sections were stained with PECA/CD31 [1:100, Pharmingen], α-smooth muscle actin (Sigma), or N-cadherin [BD Biosciences] followed by Alexa 488 goat anti-mouse and Texas red goat anti-rat in 5% nonfat dry milk in PBS. Confocal images were obtained using a Zeiss LSM510 confocal microscope. Quantitation of N-cadherin localization was performed using the MetaMorph software. A constant background value was subtracted from each image. Intensities were quantified by averaging fluorescence intensity along a line drawn following the EC/EC or EC/VSMC boundary on confocal images.

For N-cadherin-GFP localization, HUVEC were transiently transfected with pNcad-GFP [Mary et al. 2002] kindly provided by Dr. Gauthier and live-cell imaging was done by scanning at 1-min intervals using a Zeiss LSM510 confocal microscope. Time series images were stored as a movie file at 8 frames/sec. For EB1-GFP [kindly provided by Dr. Piehl, Department of Biological Sciences, Lehigh University, Bethlehem, PA] [Piehl et al. 2004], transfected HUVECs were scanned at 15-sec intervals and stored as 15 frames/sec. For coculture imaging one cell type was labeled with FM4-64 [Molecular Probes] to distinguish it from the other cell types. For the study of dorsal ruffle formation cells were fixed and stained with antibodies followed by incubation with Dif-C18 (1 μg/mL, Molecular Probes, Inc.) for 30 min. Co-localization of N-cadherin (mouse monoclonal, BD Biosciences) with connexin43 [rabbit polyclonal, Zymed] or Tie2 (rabbit polyclonal, Santa Cruz Biotechnology, Inc.) was performed using Alexa 546 (goat anti-mouse) and Alex 488 (goat anti-rabbit). Optical z-slices (0.5 µm) were collected and viewed using the LSM510 browser.

**N-cadherin–Fc-bead adhesion assay**

pCECH-NcadFc [construct encoding chicken N-cadherin ectodomain-mouse IgG Fc chimera] was kindly provided by Dr. Mege [Signalisation et Differentiation Cellulaires dans les Systèmes Nerveux et Musculaire, U440 INSERM/UPMC, Institut du Fer a Moulin, Paris, France] [Lambert et al. 2000]. NcadFc protein was collected from AIM-V medium of HEK293 cells transfected with pCECH-NcadFc using protein G beads. Protein [200 μg] was eluted and rebound to goat anti-mouse Fc IgG (200 μg, Jackson Immunoresearch)-coated polystyrene latex sulfate beads [1 μm, 2 × 10⁹; Interfacial Dynamics]. Beads were placed on top of cells for 1 h in the presence or absence of S1P. Nocodazole [10 μM for 10 min or 2 μM for 1 h] or cytochalasin D [1 μM for 10 min] were incubated with the cells before fixation and analysis. N17Rac, N19Rho, and pTet adenoviruses were gifts from Dr. Sessa [Department of Pharmacology, Yale University School of Medicine, New Haven, CT] [Lambert et al. 2000]. HUVECs were infected with dominant negative viruses [100 M.O.I] along with pTet (5 M.O.I) in the presence of 2 μg/mL tetracycline. Cells were recovered and induced to express N17Rac or N19Rho by removing tetracycline overnight. Beads were visualized by labeling with Alexa conjugated anti-mouse Fc antibodies and their numbers were quantified on a single cell basis with the ImageJ version 1.30 software.

**Ex vivo rat aorta model of angiogenesis and vessel maturation**

Aortic rings obtained from 5- to 10-week-old Sprague-Dawley male rats [Charles River Laboratories] were embedded in type I collagen gels and transferred to 24 wells, each containing 0.5 mL serum-free endothelial basal medium as described [Nicosia and Ottinetti 1990]. The growth medium was changed three times a week starting from day 3. siRNA against N-cadherin was complexed with liposome and overlaid on aortic rings embedded in collagen gel as 1 μM every 2 d. As a control scrambled siRNA was used. After 96 h, cultures were treated with 3-bromo-2’-deoxy-uridine (BrdU), for 1 h and gels were embedded in OCT.
and cryosectioned. Ten-micron sections were stained for PECAM, α-smooth muscle actin, or anti-BrdU and images were scanned using a Zeiss LSM510 confocal microscope. Z-serial images were three dimensionally reconstructed using IMARIS software (Bitplane).

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