Vascular Integrity Mediated by Vascular Endothelial Cadherin and Regulated by Sphingosine 1-Phosphate and Angiopoietin-1

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Development of blood vessels is coordinated by angiogenesis and stabilization of vascular endothelial cells (ECs). The vascular network is established during embryogenesis to supply oxygen and nutrients to the tissues and organs. However, after cardiac or peripheral ischemia is caused by occlusion of the vessels, new vessels must be formed to rescue the ischemic tissues. Many angiogenic growth factors and chemokines are produced in the ischemic tissue to induce angiogenic sprouting of preexisting vessels. Branched vessels must be again restabilized to form mature vessels that deliver blood to the tissues. To this end, vascular EC–cell adhesion is tightly regulated by cell–cell adhesion molecules and extracellular stimuli that activate G protein-coupled receptors and receptor tyrosine kinases exclusively expressed on vascular ECs. This review spotlights the recent studies of vascular endothelial cadherin and of sphingosine 1-phosphate signaling and angiopoietin-Tie signaling. 

Key Words: Angiogenesis; Angiopoietin; Sphingosine 1-phosphate; Stabilization; Vascular endothelial cadherin

How is Vascular Integrity Regulated?

Blood Vessels Regulate Delivery of Blood and Nutrition by Controlling Blood Flow and Permeability

Neovascularization is required for developmental (physiological) and pathological angiogenesis. The latter is found in the ischemic heart, peripheral arterial disease and tumors. Thus it is important to assist neovascularization in ischemic diseases and to block angiogenesis in cancer. The vascular network of the body maintains the circulation of blood and nutrients necessary for the peripheral tissues and organs by relaxing and constricting autonomously or non-autonomously. Many of the molecules produced by endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), which affect relaxation or contraction of blood vessels in both an autocrine and paracrine manner, have been identified. Among them, sphingosine 1-phosphate (SIP) released from vascular ECs stimulates S1P receptors on the ECs to induce vascular relaxation and those on the VSMCs to induce vascular contraction. Vascular ECs produce nitric oxide (NO) to induce the relaxation of VSMCs. The NO-dependent cGMP-PKG signal modulated by phosphodiesterase in VSMCs is central to vascular relaxation.

On the other hand, ECs synthesize vascular contraction factors, including the metabolites of arachidonic acids. Vascular contraction and relaxation are mainly regulated by the activity myosin light-chain kinase (MLC-K) controlled by the intracellular increase/decrease in Ca²⁺ and Rho kinase in the signaling provoked by G protein-coupled receptors (GPCR). Recently, it has been also shown that oxidative stress affects vascular cells. In order to deliver nutrients and blood, vessels not only relax/contract but also loosen/tighten the cell–cell adhesions, which are constituted by endothelial-specific and common adhesion molecules. Structural characteristics of vascular ECs determine the supply of the molecules in the blood vessels across the monolayer of ECs. How the molecules pass through this barrier has been extensively studied. The concentration of molecules inside or outside of the monolayer is determined by transcellular transport and paracellular permeability. The transcellular transport of the molecules occurs in the fenestrated capillaries, whereas the paracellular pathway is via the continuous capillaries. The surface of the endothelial cellular that consists of glycocalyx sieves the molecules that will be subjected to the transcellular or paracellular pathway. The presence of basement membrane beneath the endothelial monolayer affects the diffusion of the molecules that pass the endothelial layer. ECs are attached to the extracellular matrix (ECM) via integrin receptors to form an EC–ECM adhesion, which is involved in both the transcellular and paracellular pathways. In clear contrast, the cell–cell adhesions directly control the paracellular pathway (Figure 1).

Roles of Adhesion Molecules Expressed on Vascular ECs in Extravasation of Blood Cells

The intercellular adhesions between vascular ECs mainly constitute adherens junctions and tight junctions. Vascular ECs express endothelial-specific adhesion molecules: vascular endothelial cadherin (VE-cad), and platelet and endothelial adhesion molecule-1 (PECAM-1). In addition to these adhesive molecules, junctional adhesion molecule (JAM), claudin-5 and nectin are involved in the formation of vascular integrity.
of cell–cell contacts.\textsuperscript{16–18} VE-cad constitutes adherens junction, whereas claudin-5, JAM and ESAM form tight junctions. Recently, VE-cad was shown to transcriptionally upregulate claudin-5 by inducing the nuclear export of β-catenin (\textit{β-ctn}), which is mediated by the formation of a FoxO-1 and \textit{β-ctn} complex outside of the nucleus.\textsuperscript{19} PECAM-1 localizes in the region outside of the adherens and tight junctions\textsuperscript{20} (Figure 2).

The interendothelial cell–cell adhesions regulate the transendothelial migration of blood cells into the intersti-
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Control of Vascular Integrity

Permeability Regulating Factors

Increased vascular permeability is found in inflammation, hypoxia and thrombosis. Under these conditions, vascular permeability is either enhanced or reduced by extracellular stimuli that activate cell surface receptors, tyrosine kinases and GPCRs. Vascular endothelial growth factor (VEGF) is a vascular permeability increasing factor, whereas Angl activates Tie2 receptor tyrosine kinase and counteracts the VEGF–VEGFR-2 signal to maintain vascular integrity.

Several permeability-increasing factors are induced through GPCR activation. Thrombin is concentrated in clots and released into the blood to activate PAR-1, which is activated when its amino-terminus is cleaved upon thrombin stimulation, and it then signals to Gq and G12/13 proteins to increase permeability. Gq and G12/13 activation leads to increased intracellular Ca2+, which enhances activation of calmodulin kinase (CaM-K)/MLC-K, leading to activation of Rho–Rho kinase to reduce MLC phosphatase. Both signals result in actin–myosin-dependent contraction of vascular ECs. Bradykinin produced from kininogen in the blood acts on the Gq/i protein-coupled B1 and B2 receptors expressed on ECs to increase permeability. In contrast to thrombin, bradykinin induces vascular permeability independently of MLC-K and Rho kinase. Histamine released from mast cells also transiently increases permeability, compared with thrombin, by activating H1 receptors expressed on ECs. Platelet-activating factor augments vascular permeability independently of the actin–myosin contraction, similar to bradykinin (Figure 3).

VE-Cad Regulates Barrier Function in ECs

VE-cad and N-cadherin belonging to the classical cadherin family that are expressed on the endothelium and undergo Ca2+-dependent homophilic trans- and cis-interactions. VE-cad accumulates at the interendothelial cell–cell junctions, whereas N-cadherin is mostly located between ECs and smooth muscle cells. In contrast, there is a report of localization of N-cadherin at the interendothelial junctions. Similar to other classical cadherins, VE-cad is a single-membrane-spanning molecule that contains 5 extracellular cadherin domains, a transmembrane domain and a cytoplasmic domain that binds p120 catenin (p120ctn) and β-catenin at the juxtamembrane domain and the carboxy terminal domain, respectively.

The expression of VE-cad is regulated by many steps: shedding, endocytosis, synthesis, transport and stabilization at the cell–cell contacts (Figure 4). The extracellular domain of VE-cad is detected in the culture medium from apoptotic ECs, indicating shedding of VE-cad. Recently, the metalloprotease-disintegrin, ADAM9, was reported to potentially cleave VE-cad.

VEGF induces endocytosis of VE-cad through the phosphorylation of VE-cad at Ser 665. Activation of Rac by Vav2 in VEGF-stimulated cells leads to PAK-dependent phosphorylation of Ser at 665, resulting in endocytosis of VE-cad in a manner dependent on β2-arrestin2. Src tyrosine phosphatases (SHP1 and SHP2) are recruited to the juxtamembrane domain of VE-cad, which is known to control endocytosis of VE-cad. In addition, MLC-PPase, myosin light chain phosphatase; CaM-K, calmodulin kinase; MLC-K, myosin light chain kinase.

Figure 3. Stabilization (indicated by black font) and destabilization (indicated by red font) is controlled by receptor tyrosine kinases and G protein-coupled receptors expressed on the vascular endothelial cells. Activation of sphingosine 1-phosphate receptor 1 (S1P1) and Tie2 enhances cell–cell adhesions, whereas that of VEGF receptor and PAR-1 receptor destabilizes the cell–cell adhesion. Gi-mediated signal downstream of S1P1 exerts a vasculoactive protective action via endothelial nitric oxide synthase, while Gq- and G12/13-induced signal promotes the contraction of endothelial cells, thereby physically dissociating cell–cell contacts. MLC-PPase, myosin light chain phosphatase; CaM-K, calmodulin kinase; MLC-K, myosin light chain kinase.
kinase is involved in the Vav2–Rac–PAK signal-mediated phosphorylation of VE-cad. Although phosphorylation of the cytoplasmic domain of VE-cad is accompanied by an increase in vascular permeability in vitro and in vivo, it has not been explored whether tyrosine-phosphorylated VE-cad is prone to endocytosis. Src is also involved in tyrosine phosphorylation of VE-cad.

VE-cad associates with VEGFR-2 (Flk-1) and reduces the internalization of VEGFR-2. The significance of the association of VE-cad with VEGFR-2 is confirmed by mice lacking the entire VE-cad and those lacking its cytoplasmic domain, although the precise mechanism of VEGF-mediated internalization of VE-cad has yet to be clarified.

The VE-cad promoter has been isolated and is used as an endothelial-specific promoter. There are 2 conserved Ets binding sites in the 5' region (within −200bp) of the transcription initiation site of the VE-cad gene (CDH5). Chromatin immunoprecipitation assay confirms that Erg is capable of binding these 2 sites. Perturbation of Erg using antisense oligonucleotides reduces the expression of VE-cad in HUVECs, indicating the involvement of Erg in the transcriptional regulation of VE-cad. There are 6 potential and putative hypoxia response elements, suggesting the regulation of VE-cad expression by hypoxia-inducible factors (HIF-1α and HIF-2α). Although CDH5 is not responsive to hypoxia, HIF-2α, but not HIF-1α, activates the promoter of VE-cad. Serum response factor (SRF) is also reported to be required for the expression of VE-cad, as confirmed by the depletion of SRF in the vascular ECs of mice. SRF binds to CArG box found in the regulatory regions of CDH5.

The cytoplasmic domain of VE-cad is important for the trafficking and stabilization of VE-cad because it provides the binding sites for p120ctn and β-ctn. The amount of p120ctn determines a set point mechanism that regulates cadherin expression levels by controlling the internalization and degradation of VE-cad. Reduction of p120ctn by siRNA results in decreased expression of endogenous VE-cad. Forced expression of p120ctn reduces the clathrin-mediated endocytosis of VE-cad. The essential role of the association between p120ctn and VE-cad is supported by evidence that overexpression of the juxtamembrane domain of VE-cad reduces VE-cad expression and barrier function.

Furthermore, p120ctn regulates the transendothelial migration of leukocytes by altering the tyrosine phosphorylation of VE-cad. Conversely, phosphorylation of VE-cad at Tyr658 and Tyr731 inhibits binding of p120ctn to VE-cad. Phosphorylation of the 2 tyrosines correlates with a reduction in the barrier function of ECs. Another study reports that Golgi-associated cPLA2α regulates the transport of VE-cad, as well as claudin-5, from the Golgi apparatus to the cell–cell junction.

The β-ctn binding to E-cadherin is well known as linking E-cadherin to actin via α-catenin (α-ctn) to stabilize E-cadherin-mediated cell–cell adhesion. Thus, similar to E-cadherin, VE-cad is thought to be linked to actin fibers to maintain the integrity of endothelial adhesions. Depletion of β-ctn in the ECs of mice results in defective vascular patterning, suggesting the critical role of β-ctn in interendothelial adhesions. Therefore, stabilization of VE-cad depends on the link between VE-cad and actin. However, this classical model is challenged by the Nelson and Weis groups. They claim that α-ctn does not connect actin fibers to E-cadherin and propose a dynamic model instead of the classical static model. In addition, phosphorylation of β-ctn as well as VE-cad affects the binding of β-ctn to VE-cad. There are several phosphatases that dephosphorylate the molecules participating in the regulation of cell–cell adhe-
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Thrombin induces phosphorylation of SHP2 and its dissociation of VE-cad, resulting in the phosphorylation of β-ctn and a reduction of the amount of β-ctn that binds to VE-cad. The requirement of vascular endothelial protein tyrosine phosphatase (VE-PTP) has been demonstrated. Downregulation of VE-PTP enhances EC permeability and inhibits VE-cad adhesion. The essential substrate of VE-PTP in VE-cad-dependent cell–cell adhesions is plakoglobin, not β-ctn. DEP-1/CD148 is reported to be required for VE-cad-dependent inhibition of VEGFR-2 activation.

The cellular actin cytoskeleton that determines cell shape is regulated by the Rho-family GTPases (Rho, Rac and Cdc42). Indeed, Rho-family GTPases are involved in cadherin-mediated cell–cell adhesion by controlling the actin-based cytoskeleton. Besides the Rho-family GTPases, Rap1, a small GTPase, is well known for stabilizing the VE-cad-mediated cell–cell adhesion.

Stabilization of VE-Cad at the Cell–Cell Contact by Rap1-Mediated Signal

We have previously shown that MAGI-1 binding to β-ctn recruits PDZ-GEFI, a guanine nucleotide exchange factor (GEF) for Rap1, thereby activating Rap1 at the interendothelial junctions upon engagement of VE-cad. My and others further demonstrate that the cAMP–Epac (another GEF for Rap1)–Rap1 signal stabilizes the VE-cad-mediated cell–cell adhesion. Although cAMP-increasing stimuli are known to stabilize the cell–cell adhesion via protein kinase A (PKA), Epac instead of PKA is critical for cAMP-induced stabilization. Accumulation of VE-cad at the cell–cell contacts is increased by an Epac-specific activator, 8-pCPT-2′-O-Me-cAMP (known as 007), as well as forskolin in vitro and in vivo. 007 not only reduces EC permeability, but also inhibits transendothelial migration of leukocytes.

Although the downstream effectors of Rap1 in the stabilization of VE-cad are not fully understood, several potential signaling pathways have been suggested. Cerebral cavernous malformation (CCM) is associated with defective endothelial junctions. There are 3 genes related to CCM: CCM1, CCM2 and CCM3. CCM1/KRIT1 (K-Rev1 interaction trapped gene 1), a Rap1-binding protein, is reported to be involved in Epac1/Rap1-regulated permeability of EC–cell junctions. KRIT1 localizes at the cell–cell contacts and associates β-ctn, thereby being indirectly connected to VE-cad. CCM2 functions as a scaffold protein and forms complexes with CCM1 and CCM3. CCM1 might connect the heart-of-glass (Heg)-mediated signal to CCM2 to maintain vascular structure, as well as VE-cad-β-ctn.
monitor the dynamics at the junctions. We found that for (GFP) (VE-cad-GFP and PECAM-1-GFP, respectively) to VE-cad or PECAM-1 tagged with green fluorescence protein recovery after photobleaching (FRAP) experiment using not linked to actin. Thus, my group used a fluorescence be claimed by comparing VE-cad with PECAM-1, which is the importance of the link between VE-cad and actin can not be contradicted. The role of affadin, which can bind to Rap1, nectin, and zonula occludens-1 (ZO-1), has not been clearly investigated in the Rap1-mediated signal, although its role in the EC–ECM has been characterized.77 Rhō-family GTPases are regulated by other molecules localized at the cell–cell contacts. p120ctn and RhoA mutually inhibit their activities by binding each other.78 p120ctn balances the activity of Rho and Rac by binding to p190RhoGAP at the cell–cell contacts when p190RhoGAP is recruited to the cell–cell contacts upon growth factor stimulation.79

In the light of cortical actin-dependent stabilization of VE-cad, how Rap1 regulates VE-cad stabilization at the cell–cell contacts has been investigated. Although the static linkage of cadherin and actin is challenged by the Nelson and Weis groups,70 cell–cell contact-dependent engagement of nectin induces the activation of Rap1 and subsequent activation of Rac and Cdc42 via Vav2 and FRG, respectively.15 The role of affadin, which can bind to Rap1, nectin, and zonula occludens-1 (ZO-1), has not been clearly investigated in the Rap1-mediated signal, although its role in the EC–ECM has been characterized.77 Rhō-family GTPases are regulated by other molecules localized at the cell–cell contacts. p120ctn and RhoA mutually inhibit their functions by binding each other.78 p120ctn balances the activity of Rho and Rac by binding to p190RhoGAP at the cell–cell contacts when p190RhoGAP is recruited to the cell–cell contacts upon growth factor stimulation.79

In the light of cortical actin-dependent stabilization of VE-cad, how Rap1 regulates VE-cad stabilization at the cell–cell contacts has been investigated. Although the static linkage of cadherin and actin is challenged by the Nelson and Weis groups,59 the importance of the link between cadherin and actin cytoskeleton has not been contradicted. The importance of the link between VE-cad and actin can be claimed by comparing VE-cad with PECAM-1, which is not linked to actin. Thus, my group used a fluorescence recovery after photobleaching (FRAP) experiment using VE-cad or PECAM-1 tagged with green fluorescence protein (GFP) (VE-cad-GFP and PECAM-1-GFP, respectively) to monitor the dynamics at the junctions. We found that forskolin induces cortical actin bundling parallel to the cell–cell contacts. The mobile fraction of VE-cad was decreased upon forskolin stimulation, while that of PECAM-1 was unchanged, suggesting a significant link between actin and cell adhesion molecules for the stabilization of adhesion molecules at the cell–cell contacts when the formation of cortical actin bundling is augmented (unpublished data). The precise molecular mechanism of how Rap1 regulates the stabilization of VE-cad at the cell–cell contact needs to be clarified (Figure 5).

**Ang1/Tie2-Mediated Integrity of Vascular ECs**

Ang1 activates Tie2 receptor tyrosine kinase and counteracts the VEGF-VEGFR-2 signal to maintain vascular integrity.28,29 Another angiopoietin, Ang2, is thought to compete with Ang1 and induce angiogenesis.80 Ang1 strengthens the PECAM-1- and VE-cad-mediated intercellular junctions by inducing the dephosphorylation of PECAM-1 and VE-cad and by inhibiting TNF-α-induced transmigration of leukocytes.81 We and others have demonstrated that Ang1 localizes Tie2 to the intercellular junctions by bridging Tie2.82,83 Trans-associated Tie2 preferentially activates AKT and reduces permeability. VE-PTP is also recruited to the cell–cell junctions when stimulated by Ang1, suggesting a potential role in reducing the permeability mediated by enhanced accumulation of VE-cad. Antibody against VE-PTP can mimic depletion of VE-PTP and induces activation of Tie2. Thus, the association of VE-PTP with Tie2 appears to be very important for the regulation of endothelial stabilization and proliferation.84 We further extended our studies of the function of trans-associated Tie2 at the cell–cell contacts. Analysis of gene expression by cDNA array enabled identification of the responsible molecules that promote vascular quiescence, 1 of them being Krüppel-like factor 2 (KLF2).82 KLF2 is a zinc finger transcription factor that belongs to the KLF family and is expressed in vascular...
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**References**


Vascular EC integrity is important for protecting the vasculature from thrombosis, atherosclerosis and abnormal angiogenesis. Because the ideal vasculature is organized during embryonic development, established blood vessels need to be maintained to keep blood flowing smoothly around the body. In this review, I have focused on the molecules that are central to the stability of vascular ECs. Other vascular stabilization factors besides Ang1 and S1P will be identified to extend our knowledge of vascular integrity. Destabilization of cell–cell contacts is required for preexisting vessels to promote the angiogenesis that should be re-stabilized once ischemia is improved. Therefore, it is necessary to understand the stabilization and destabilization cycle in the vasculature under both pathological and physiological conditions. We, as cardiologists, are expected to develop new therapeutic strategies to rescue and reverse the damaged heart by regenerative medicine. We need to think about the regeneration of healthy vasculature, as well as the myocardium, to regenerate the heart. Thus, it is important for us to instigate understanding of how we can control both regeneration and maintenance of preexisting tissues.

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**SIP Regulates Integrity of ECs**

SIP is thought to be released from activated platelets and erythrocytes. Its receptors, S1P1–S1P5, are GPCR and expressed in various tissues, including vascular ECs and VSMCs. S1P1 is coupled to Gi and induces PI3K-dependent eNOS and Rac activation, whereas S1P2 and S1P3 are coupled to Gi, Gq, and G12/13. Activation of Gq and that of G12/13 results in phosphorylation of PLC and subsequent actin–myosin-dependent contraction via the phospholipase C/PLC/Ca²⁺/CaM kinase signal and RhoA–Rho kinase signal, respectively.

Vascular integrity controlled by SIP is dependent on the distinctive cytoskeleton. SIP-mediated enhancement of the EC barrier function is explained by the phosphorylation of cortactin and its binding to MLC-K. ZO-1 is essential for the SIP-regulated barrier integrity of ECs, as evidenced by the fact that depletion of ZO-1 leads to loss of the SIP-enhanced barrier function. The association of VE-cad with β-catenin upon S1P stimulation regulates the cortical actin rearrangement that stabilizes the cell–cell junction.

Plasma S1P-less mice in which sphingosine kinases (I and II) are knocked out show vascular leakage, suggesting the mechanism of S1P1-regulated vascular integrity of ECs in vivo. Thus, maintenance of vascular EC integrity is regulated by SIP without activation of platelets.

From which cells is SIP released? SIP is synthesized from sphingosine localized in the plasma membrane by sphingosine kinase I and II in the cytoplasm. Therefore, SIP produced in the cells must be released from inside to outside of the cells via transporters that activate SIP receptors. There are 3 ABC transporters, ABC1, ABCA1, and ABCA7, which might function as the SIP transporter. It is still controversial whether these transporters function in vivo to maintain vascular integrity. My group recently identified a novel SIP transporter, Spinster2 (Spns2), by screening zebrafish mutants that show cardiac bifida. The Spns family consists of Spns1, Spns2 and Spns3. The mutants showing cardiac bifida harbored a R153S mutation of Spns2, indicating that it was the gene responsible. Stainier’s group previously reported that deletion of S1P2 causes cardiac bifida in both zebrafish and another mutant fish. By analyzing the latter, they also demonstrated that the gene responsible for the phenotype was a mutation of Spns2. We assumed that Spns2 might function as a SIP transporter because it appears to span the membrane 12 times according to its amino acid sequence. Therefore, we investigated the export activity of SIP using cells expressing wild-type Spns2 and mutant Spns2 (R153S) and found that Spns2 functioned as a transporter of SIP. Collectively, SIP released from vascular ECs via Spns2 might activate S1P1 expressed on vascular ECs to maintain vascular integrity (Figure 6).

**Conclusion**

ECs.

The importance of KLF2 for the stability of blood vessels is confirmed by deletion of KLF2. One can speculate that KLF2 is a vascular protective transcription factor because it upregulates endothelial NO synthase (eNOS), natriuretic peptides and downregulates VCAM-1, E-selectin, VEGFR-2, and endothelin-1.

My group delineated the signaling downstream of activated Tie2 at the cell–cell contacts. Upregulation of KLF2 is dependent on MEF2, but not ERK5, which is regulated by the phosphatidylinositol 3-kinase (PI3K)-AKT signal. Attachment of monocytes to cultured ECs stimulated with VEGF was impeded by Ang1. This inhibition was reversed by the depletion of KLF2, indicating that the inhibitory effect of Ang1 on inflammatory cell adhesion to ECs is regulated by KLF2. Therefore, we conclude that Ang1-dependent stability of ECs depends on KLF2 (Figure 6).

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**References**


