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Review

Vascular Endothelial Cadherin-mediated Cell-cell Adhesion Regulated by a Small GTPase, Rap1

Shigetomo Fukuhra, Atsuko Sakurai, Akiko Yamagishi, Keisuke Sako and Naoki Mochizuki*

Department of Structural Analysis, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

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Vascular endothelial cadherin (VE-cadherin), which belongs to the classical cadherin family, is localized at adherens junctions exclusively in vascular endothelial cells. Biochemical and biomechanical cues regulate the VE-cadherin adhesive potential by triggering the intracellular signals. VE-cadherin-mediated cell adhesion is required for cell survival and endothelial cell deadhesion is required for vascular development. It is therefore crucial to understand how VE-cadherin-based cell adhesion is controlled. This review summarizes the inter-endothelial cell adhesions and introduces our recent advance in Rap1-regulated VE-cadherin adhesion. A further analysis of the VE-cadherin recycling system will aid the understanding of cell adhesion/deadhesion mechanisms mediated by VE-cadherin in response to extracellular stimuli during development and angiogenesis.

Keywords: Adherens junction, Permeability, Rap1, Vascular endothelial-cadherin (VE-cadherin)

Introduction

Vascular endothelial cells aid in the regulation of blood flow for the supply of nutrients to the tissues. Morphologically, vascular endothelial cells make cell-to-cell contact, not only with the neighboring endothelial cells, but also with supporting pericytes in the capillaries. In addition, they are supported by the extracellular matrix and inner basal lamina (Davis and Senger, 2005). Vascular endothelial cell-cell adhesions are organized mainly by adherens junctions (AJs), tight junctions (TJs), and gap junctions (GJs) as other epithelial cell-cell adhesions (Fig. 1). The most striking difference between endothelial cell-cell contacts and epithelial cell-cell contacts is that the junctions are intermingled in the former while TJ-AJ-GJ is organized from the apical to base side in the latter. To date, the function of specific AJ-, TJ-, and GJ-constituting molecules within endothelial cells have extensively analyzed. It is essential for endothelial cells to maintain cell-cell adhesion in order to keep morphological integrity and quiescence.

The extracellular stimuli loosen cell-cell contacts prior to promoting proliferation of vascular endothelial cells. Such includes vascular endothelial growth factor (VEGF) and its related factors (reviewed in (Yancopoulos *et al.*, 2000) and (Gale and Yancopoulos, 1999)). VEGF was initially described as a vascular permeability factor, as its name indicates (Keck *et al.*, 1989). On the contrary, Angiopoietin (Ang) stabilizes the cell-cell contacts and reduces vascular permeability via activation of the Tie2 receptor tyrosine kinase. Both VEGF-VEGF receptor (VEGF-R), and Ang-Tie2 signaling are required for embryonic vascular development (Shalaby *et al.*, 1995; Fong *et al.*, 1995; Suri *et al.*, 1996; Gale *et al.*, 2002). Another receptor tyrosine kinase system, ephrin-Eph tyrosine kinase, is also indispensable for vascular development. Ephrin is anchored by GPI (ephrin-A family), or single spanning (ephrin-B family), and therefore cell-cell contacts can trigger Eph receptor activation, resulting in either a repulsive or adhesive action between vascular endothelial cells. Thus, ephrin-Eph is believed to induce the motility of vascular endothelial cells and to determine the lineage of arterial endothelial cells and venous endothelial cells (Adams and Klein, 2000; Nagashima *et al.*, 2002).

In this review, we highlight the recent progress in studies concerning the adhesion molecules of AJ and TJ in vascular endothelial cells, particularly vascular endothelial cadherin (VE-cadherin)-based AJ formation. In addition, we present our recent findings, which suggest that the balance between VE-cadherin trafficking and the stabilization of assembled VE-cadherin regulates the integrity of the endothelial cells. We further suggest the importance of the trafficking regulated by extracellular stimuli, although recent studies have illuminated the mechanism of assembly and disassembly of adhesion molecules.

*To whom correspondence should be addressed.
Tel: 81-6-6833-5012 ext 2508; Fax: 81-6-6835-5461
E-mail: nmochizu@ri.ncvc.go.jp

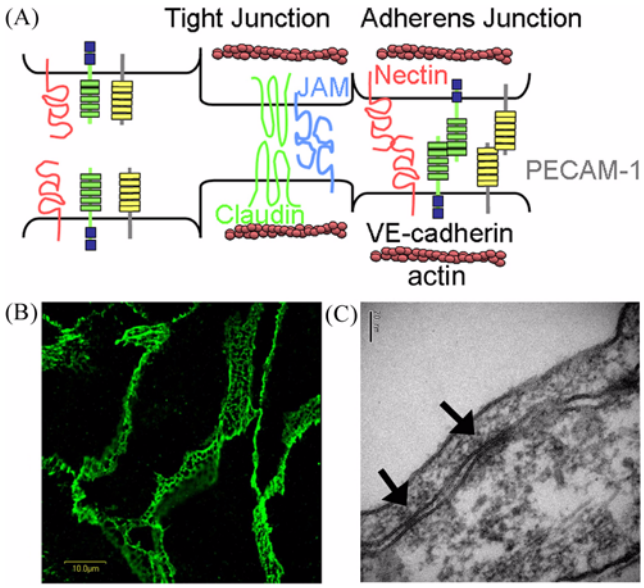

Fig. 1. Structural characteristic of endothelial cell junctions. (A) Vascular endothelial junction molecules. VE-cadherin, Nectin, and PECAM-1 are localized at the adherens junction, whereas JAM and Claudin are at the tight junction. (B) Cultured human umbirical vein endothelial cells (HUVECs) are immunostained with anti-VE-cadherin antibody and visualized by fluorescence (green)-conjugated secondary antibody. Note that VE-cadherin are found belt-shaped on the overlapped peripheral membrane of endothelial cells, suggesting that AJs and TJs are intermingled. (C) Electron microscope image of overlapped endothelial cells. Arrows denote the adherens junctions.

Junctional molecules of vascular endothelial cells

AJs consist of VE-cadherin (Lampugnani *et al.*, 1995), Nectin-2 (Reymond *et al.*, 2004), and platelet and endothelial cell adhesion molecule-1 (PECAM-1) (Fig. 1) reviewed in (Dejana, 2004). VE-cadherin belongs to the classical cadherin superfamily and exhibits cis and trans homophilic association via external 5 cadherin domains in a Ca^{2+}-depedent manner (Chappuis-Flament *et al.*, 2001). It spans the plasma membrane and binds to p120catenin (p120ctn) and β-catenin (β-ctn) in the proximal and distal cytoplasmic domain, respectively. Because β-ctn directly associates with α-ctn, which is connected to cortical actin, VE-cadhrerin is supported by cytoskeletal actin. However, this scheme has been recently questioned by two groups (Yamada *et al.*, 2005; Drees *et al.*, 2005). These two groups suggest a more dynamic role of α-ctn in cortical actin assembly near the cadherin-based adhesion. Likewise, nectin is linked to cortical actin fibers by binding to afadin, which is associated with actin fibers. PECAM-1 is also reported to be associated with β-ctn. Thus, VE-cadherin, nectin, and PECAM-1 may be lined by cortical action filaments. Other cadherin family members, N-cadherin and VE-cadherin2 are reported to be expressed in vascular endothelial cells (Salomon *et al.*, 1992; Telo' *et al.*, 1998). It is not yet confirmed whether N-cadherin is localized to the inter-endothelial cell-cell contacts (Salomon *et al.*, 1992; Navarro *et al.*, 1998; Luo and Radice, 2005). Although VE-cadherin2 is a single transmembrane protein like VE-cadherin and N-cadherin, it does not contain catenin-binding sites in the cytoplasmic domain. VE-cadhrin2-deficient mice do not exhibit gross vascular developmental abnormality. Thus, the function of VE-cadherin2 remains to be analyzed.

Nectins and cadherins cooperatively function for the formation of AJ. However, there is a striking difference between nectins and cadherins upon homophilic association. Cadherin, but not nectin, requires Ca^{2+} for their homophilic association. The research team of Takai demonstrated that the c-Src-Crk-C3G-Rap1 signaling, triggered by nectin engagement upon cell-cell contact, is important for AJ formation (Fukuyama *et al.*, 2005). Furthermore, they demonstrated that activated Rap1-afadin complex binds to p120ctn, thereby regulating cadherin-based AJ formation. Molecular regulation of the nectin family molecules has been extensively studied and reviewed by Takai's group (Takai and Nakanishi, 2003; Sakisaka and Takai, 2004).

PECAM-1 has six external immunoglobulin-like domains, a single transmembrane domain, and a cytoplasmic domain (Newman and Newman, 2003). The cytoplasmic domain has an ITIM motif that provides a binding site for SHP2 (Jackson *et al.*, 1997; Masuda *et al.*, 1997). Like that of other cell-cell adhesion molecules, PECAM-1-to- PECAM-1 bonding is achieved through homophilic trans-interaction. Therefore, PECAM-1 engagement is thought to provoke intracellular signaling via adaptor/docking molecules including SHP2, SHP1, PLCg, and Grb2 (reviewed in Newman and Newman, 2003). Interestingly, PECAM-1 may be involved in the mechanotransduction pathway together with VE-cadherin (Osawa *et al.*, 2002; Newman and Newman, 2003).

TJs are made up of junctional adhesion molecule (JAM) family members, endothelial cell-selective adhesion molecules (ESAM), occuludin, claudin (1, 5, and 12) and nectin (reviewed in (Dejana, 2004)). Interestingly, nectin is localized to within AJs and TJs. JAM, ESAM, claudin, and occudin are associated with zona occludens-1 (ZO1) (Gumbiner *et al.*, 1991; Wong and Gumbiner, 1997; Tsukita *et al.*, 2001; Bazzoni, 2003). Because ZO1 binds to filamentous actin, these TJ molecules are linked to the actin cytoskeleton. Other PDZ domain-containing molecules, such as MAGUK with an inverted domain structure-1 (MAGI-1) and ZO2, are capable of associating with the intracellular C-terminal region of JAM family members (Shoji *et al.*, 2000; Laura *et al.*, 2002; Wegmann *et al.*, 2004).

Signaling mediated by VE-cadherin and signaling triggered by VE-cadherin engagement

Vascular endothelial cells receive biochemical signals as well as biomechanical signals (Shay-Salit *et al.*, 2002). VE-

cadherin regulates β-ctn as a transcription factor. Because the cytoplasmic domain binds β-ctn, which translocates into the nucleus where it associates with Tcf and activates transcription of multiple genes, the association of β-ctn and VE-cadherin inhibits β-ctn-mediated transcriptional activation (Caveda *et al.*, 1996).

The tyrosine residues of cytoplasmic domain of VE-cadherin are phosphorylated by Src family kinases upon tumor necrosis factor stimulation (Nwariaku *et al.*, 2004; Lambeng *et al.*, 2005). Furthermore, extravasation of blood cells induces VE-cadherin phophorylation. Subsequently, tyrosine-phosphorylated VE-cadherin provides the docking sites for multiple signaling molecules including SHP2, phosphatidylinositol 3'-kinase (PI3K) and Shc(Ukropec *et al.*, 2000; Zanetti *et al.*, 2002; Hudry-Clergeon *et al.*, 2005). In contrast, phosphorylated VE-cadherin does not bind to p120ctn and β-ctn (Potter *et al.*, 2005). Thus, phosphorylation and dephosphorylation of VE-cadherin is implicated in intracellular signaling and stabilization of cell-cell contacts.

VEGF-VEGF-R signaling regulates permeability, vascular endothelial cell proliferation, and cell survival. VE-cadherin is involved in VEGF-mediated cell signaling (Esser *et al.*, 1998; Zanetti *et al.*, 2002; Grazia *et al.*, 2003a). VE-cadherin associates with VEGF-R2 and modulates the VEGF-R2-mediated signaling (Carmeliet *et al.*, 1999; Rahimi and Kazlauskas, 1999; Weis *et al.*, 2004). VE-cadherin further involves the phosphatase DEP-1 for cell-cell contact-dependent inhibition of cell-proliferation induced by VEGF (Grazia *et al.*, 2003b). This previous line of evidence indicates that VE-cadherin is required for VEGF-R2-mediated signals at cell-cell contacts.

Classical cadherin ligation induces the activation of Rho family GTPases (Fukata and Kaibuchi, 2001; Wheelock and Johnson, 2003). VE-cadherin homophilic ligation activates Rac1 and Cdc42 (Kovacs *et al.*, 2002; Kouklis *et al.*, 2003). Cdc42 activation further stabilizes the AJs by linking VE-cadherin and α-ctn (Broman *et al.*, 2006). Given that VE-cadherin is linked to the actin cytoskeleton and that the Rho family GTPases regulate actin reorganization, VE-cadherin engagement might regulate the actin cytoskeleton. VE-cadherin inhibits cell proliferation by altering the actin cytoskeleton. This is achieved by altering, not only cell-cell contacts, but also cell-ECM contacts (Nelson and Chen, 2003).

Small GTPase Rap1-regulated VE-cadherin-dependent cell adhesion and VE-cadherin engagement-triggered Rap1 activation

In accordance with previous reports, we have reported that cAMP-Epac-Rap1 signal stabilizes adherens junction and thereby reduces cell permeability and decreases leukocyte migration (Wittchen *et al.*, 2005; Fukuhara *et al.*, 2005; Cullere *et al.*, 2005; Kooistra *et al.*, 2005). Rap1 is a small GTPase which belongs to the Ras family of proteins and is thought to antagonize Ras function by sharing Ras effector molecules such as c-Raf, RalGDS, and PI3-K. Recent data have revealed that Rap1 functions, not only as a Ras-competitor, but also as a cell adhesion regulator, particularly at the cell-ECM (Bos *et al.*, 2001; Bos *et al.*, 2003). Rap1 is activated by several guanine nucleotide exchange factors (GEF) which have regulatory motifs besides the catalytic domain.

Among GEFs for Rap1, we have focused on the effect of Epac, which is regulated by cAMP, on cell adhesion formation (Bos, 2003), because cAMP decreases cell permeability (Langeler and van Hinsbergh, 1991; Farmer *et al.*, 2001; Hippenstiel *et al.*, 2002). It has been reported that Rap1 activation is required for E-cadherin-based cell-cell contact formation (Hogan *et al.*, 2004; Price *et al.*, 2004). We and other groups have noticed that 007, a cAMP analogue (Cullere *et al.*, 2005) that directly activates Epac without activating protein kinase A, increases GTP-bound Rap1 and subsequently augments the endothelial cell barrier function. We reasoned that cAMP induces endothelial cortical actin rearrangement in a manner dependent on Rap1 activation. Although we have not identified the molecules that regulate actin cytoskeleton downstream of Rap1, increased bundling of cortical actin might support the VE-cadherin by linking VE-cadherin and actin via α- and β-ctn.

We explored the possibility that VE-cadherin engagement activates Rap1 (Sakurai *et al.*, 2006). As mentioned earlier, MAGI-1 is capable of binding to JAM. Moreover, MAGI-1 associates with β-ctn (Kotelevets *et al.*, 2005). MAGI-1 consists of 6 PSD95/ DiscLarge/ ZO-1 (PDZ) domains, a guanylate kinase domain and two WW domains flanked by the first and second PDZ domain (Dobrosotskaya *et al.*, 1997). Since PDZ domains are docking domains for PDZ-binding molecules, MAGI-1 associates with a variety molecules such as NMDA receptors, PTEN, BAI-1, mNET1, and PDZ-GEF1 (Mino *et al.*, 2000; Dobrosotskaya, 2001) (Kawajiri *et al.*, 2000) (Dobrosotskaya and James, 2000). PDZ-GEF1 is a GEF for Rap1. We hypothesized that MAGI-1-PDZ-GEF1 signal participates in the activation of Rap1 at the cell-cell contacts in vascular endothelial cells and that this signal is triggered upon cell-cell contact.

Rap1 is activated upon cell-cell contact in vascular endothelial cells as demonstrated by fluorescent resonance energy transfer (FRET)-based probe (Mochizuki *et al.*, 2001; Sakurai *et al.*, 2006). We therefore delineated the mechanism by which Rap1 is activated. VE-cadherin homophilic interaction induced Rap1 activation, because Ca^{2+}-chelating and restoring experiments showed Rap1 activation. Using FRET technique, we found that the dominant negative form of MAGI-1, which perturbs the localization of MAGI-1 to cell-cell contacts, inhibits Rap1 activation upon cell-cell contact. In addition, MAGI-1 depletion by knockdown using siRNA inhibited the Rap1 activation at the cell-cell contact. These results suggested that MAGI-1 is required for Rap1 activation.

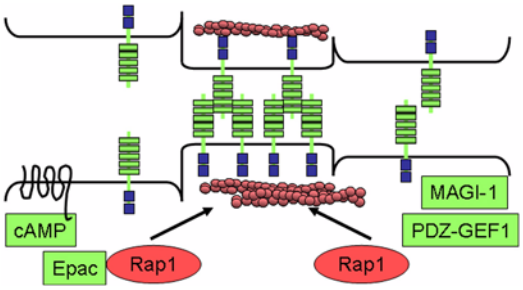

Fig. 2. Rap1 augments endothelial cell adhesion. Increase in cAMP upon G-protein-coupled receptor stimulation results in Epac-Rap1 activation, thereby inducing VE-cadherin-mediated cell adhesion. Homophilic VE-cadherin engagement also activates Rap1 via MAGI-1-PDZ-GEF1. Subsequently activated Rap1 results in rearranging actin cytoskeleton to support cell-cell adhesion.

We confirmed that MAGI-1 associates with β-ctn and PDZ-GEF1 in vascular endothelial cells.

Relocation of vinculin to cell-cell contacts from cell-ECM was hampered in MAGI-1-depleted cells, indicating that vinculin may function downstream of Rap1 for tightening cell-cell adhesion. Collectively, MAGI-1 linking β-ctn and PDZ-GEF1 is important for VE-cadherin-mediated Rap1 activation.

Rap1 activated by cAMP via Epac functions stabilizes VE-cadherin-dependent cell adhesion. Rap1 that is activated upon cell-cell contact via MAGI-1-PDZ-GEF1 also accelerates VE-cadherin-mediated cell adhesion. Thus, Rap1 regulates inside-out signal for VE-cadherin assembly. These data are summarized in Fig. 2.

Homophilic dimerization of nectin at the AJs triggers Rap1 activation via Src-Crk (Fukuyama *et al.*, 2005). Because both nectin and cadherin are present at the AJs, they coordinate AJ formation by activating Rap1. Afadin linking nectin to actin cytoskeleton appears to be a key effector molecule of activated Rap1. Although we have not yet found the direct effector of activated Rap1 upon VE-cadherin-mediated signal, both nectin- and VE-cadherin-triggered Rap1 activation results in the assembly of actin cytoskeleton. Conversely, both nectin and VE-cadherin might be supported by actin cytoskeleton.

VE-cadherin expression at the cell-cell adhesion controlled by trafficking and stabilization

VE-cadherin is not static at AJ and is endocytosed, degraded, and/or recycled by vesicular trafficking. VE-cadherin-mediated cell-cell adhesion is dynamically regulated by trafficking and stabilization at the cell-cell contacts. VE-cadherin appears to be processed by vesicular trafficking both from and to the plasma membrane, similar to E-cadherin (Bryant and Stow, 2004). However, the molecular mechanism of VE-cadherin endocytosis and exocytosis has not been completely elucidated.

Kowalczyk's group has recently reported that VE-cadherin is endocytosed in a manner dependent on clathrin (Xiao *et al.*, 2005). They reported that p120ctn inhibits VE-cadherin endocytosis. Previously, p120ctn has also been suggested to be involved in the exocytic pathway of cadherin-containing vesicles (Mary *et al.*, 2002; Peifer and Yap, 2003; Chen *et al.*, 2003). Given that the cytoplasmic domain of VE-cadherin is biochemically and structurally similar to E-cadherin, except for the absence of the di-Leu motif found in E-cadherin, VE-cadherin may enter the endocytosis pathway in a clathrin-independent manner (Akhtar and Hotchin, 2001; Paterson *et al.*, 2003).

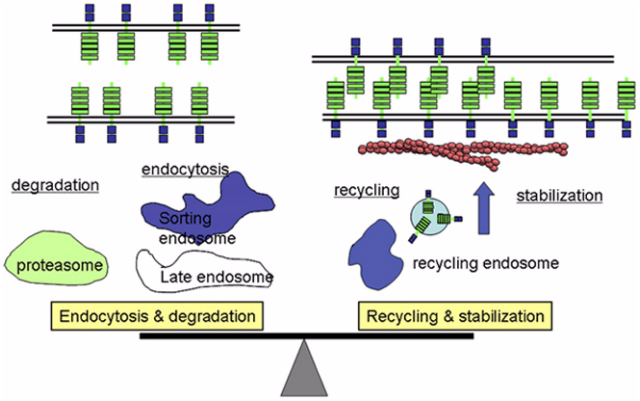

Fig. 3. VE-cadherin turnover and stabilization. Expression of VE-cadherin depends on the balance between VE-cadherin degradation/endocytosis and recycling/stabilization. Recent advance in cadherin biology has revealed the signaling cue for endocytosis and destabilization of VE-cadherin. Yet, we further need to clarify the cur for recycling and how VE-cadherin in endosomes is processed.

VE-cadherin biogenesis and turnover is not strictly analyzed yet, although half life of E-cadherin is suggested to be 2-5 hours (Gumbiner, 2000). Transcriptional regulation of VE-cadherin is not fully elucidated, although genomic organization of cadherin family genes are unraveled (Angst *et al.*, 2001). VE-cadherin is subjected to shedding by metalloproteinases (Herren *et al.*, 1998). The remaining molecules might be cleaved by γ-secretase as other classical cadherins are processed (Periz and Fortini, 2004).

Interestingly, nectin is one of the candidates as a substrate for γ-secretase (Kim *et al.*, 2002). An interesting paper demonstrated that a deficiency of Preselinin-1, a component of γ-secretase, results in abnormal vascular formation (Nakajima *et al.*, 2003). It will be interesting to study the function of the intracellular domain fragment cleaved by γ-secretase in the future. Although E-cadherin is processed by a ubiquitination pathway by Hakai (Pece and Gutkind, 2002), VE-cadherin lacks the ubiquitination site by Hakai. Thus, VE-cadherin may be degraded by alternative ubiquitination signaling.

As summarized in Fig. 3, the expression and function of VE-cadherin at the cell-cell contacts depends on the balance between internalization and stabilization. The internalized VE-cadherins are either degraded or recycled back to the membrane via trafficking and sorting signals.

Perspectives

Vascular endothelial cells have to assemble and disassemble during angiogenesis. In addition, cell adhesion is affected by the stretch induced by smooth muscle contraction and by the extravasation of blood cells. To understand how VE-cadherin-mediated cell adhesion is regulated in response to mechanical stress and biochemical signaling, we need to further study (1) what kind of stimuli controls VE-cadherin stabilization, (2) what kind of extracellular stimuli regulates the endocytosis and degradation of VE-cadherin, (3) which signals accelerate the recycling of VE-cadherin. The major unanswered question, however, relates to the cue for VE-cadherin trafficking to the plasma membrane. Recently p120ctn is involved in stabilizing VE-cadherin and trafficking of VE-cadherin (Vincent *et al.*, 2004) It will be interesting to investigate the signal that p120ctn responds in virtue of VE-cadherin regulation.

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