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van Buul, J.D.

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Chapter 7

VE-CADHERIN AND RAC1 ACT IN CONCERT TO CONTROL ENDOTHELIAL CELL-CELL ADHESION.
VE-cadherin and Rac1 act in concert to control endothelial cell-cell adhesion

Jaap D. van Buul, Eloise C. Anthony, Mar Fernandez-Borja and Peter L. Hordijk

Sanquin Research at CLB and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Abstract

VE-cadherin bridges adjacent endothelial cells and prevents vascular leakage. In order to maintain the endothelial barrier function, VE-cadherin function is tightly regulated through mechanisms that involve protein phosphorylation and cytoskeletal dynamics.

Here, we study the regulation of VE-cadherin function by using blocking antibodies that disrupt VE-cadherin homotypic binding. Incubation of endothelial cells with these antibodies results in increased tyrosine phosphorylation of β-catenin and dissociation of the VE-cadherin-catenin complex from the actin cytoskeleton. Interestingly, loss of VE-cadherin function initially induces rapid production of reactive oxygen species followed by the loss of cell-cell contact. In addition, this response involves rapid and transient activation of RhoA and prolonged activation of Rac1.

Loss of VE-cadherin function is followed by lateral membrane ruffling and spreading. Detailed analysis revealed that this membrane ruffling occurs dorsal to the retracting membrane. In line with this latter observation, a GFP-tagged version of Rac1 was found in membrane ruffles and in newly formed cell-cell junctions, where it co-localizes with VE-cadherin. Finally, use of a membrane-permeable peptide inhibitor showed that Rac1 activity is required for the loss of VE-cadherin function but also for the restoration of cell-cell adhesion.

In conclusion, these data show that loss of VE-cadherin-mediated cell-cell adhesion induces signaling which involves activation of Rho-like GTPases and
production of reactive oxygen species, and is followed by Rac-mediated spreading, which drives the restoration of cell-cell contact.

Introduction

Vascular-Endothelial Cadherin (VE-cadherin, Cadherin-5) is a transmembrane, calcium-dependent, homophilic adhesion molecule that bridges adjacent endothelial cells. Loss of VE-cadherin function results in unstable endothelial junctions and a decrease in endothelial monolayer resistance, despite the fact that several other proteins, such as claudin, occludin and PECAM-1, are also present at sites of endothelial cell-cell contact. Thus, VE-cadherin function is indispensable for the maintenance of the endothelial barrier function.

VE-cadherin is linked to the actin cytoskeleton via the armadillo-family members β- and γ-catenin that bind the actin-binding protein α-catenin [5,29]. VE-cadherin function is controlled by cytoskeletal dynamics and by protein phosphorylation events. Lampugnani and colleagues showed that tyrosine phosphorylation of VE-cadherin and associated catenins is increased in loosely confluent endothelial monolayers, whereas tyrosine phosphorylation is reduced in confluent cells [12]. Recently, a novel vascular endothelial-protein tyrosine phosphatase (VE-PTP) was shown to interact with VE-cadherin and to increase VE-cadherin-mediated barrier function [15]. In addition, Ukropec and co-workers reported that the phosphatase SHP-2 interacts with β-catenin and thereby regulates thrombin-induced changes in the endothelial barrier function [24]. The specific association of VE-PTP with VE-cadherin and SHP-2 with β-catenin provides further evidence that tyrosine phosphorylation of the VE-cadherin-catenin complex is important for the regulation of endothelial cell-cell adhesion.

In addition, the dynamics of the actin cytoskeleton also control VE-cadherin function. Our previous work has shown that VE-cadherin co-localizes with the tips of actin stress fibers at cell-cell junctions [8]. Moreover, stress fiber formation induced by thrombin or histamine is accompanied by a loss of VE-cadherin-mediated cell-cell contacts and increased permeability of the endothelial monolayers [1,16]. Thus, actin cytoskeleton-mediated contractility plays an important role in the regulation of VE-cadherin-based cell-cell junctions.
We and others have previously reported that antibody-mediated inhibition of VE-cadherin function promotes extravasation of leukocytes in vitro and in vivo [6;25]. However, to avoid vascular leakage during leukocyte extravasation or related (patho) physiological events, maintenance of VE-cadherin-mediated cell-cell adhesion is of key importance and must be tightly controlled. Although mechanisms that (in part) constitute the inside-out control of VE-cadherin function have been described, it is unknown how endothelial cells respond to reduced cell-cell adhesion in order to restore vascular integrity.

To analyze this outside-in signaling, we used antibodies that block interactions between the extracellular regions of the VE-cadherin protein and thus induce loss of VE-cadherin-mediated cell-cell contacts [3;4;8;25]. Here, we show that loss of VE-cadherin function increases the levels of tyrosine phosphorylated β-catenin and promotes the association of α-catenin to β-catenin. Moreover, loss of VE-cadherin function activates the small GTPases Rac1 and RhoA and induces membrane ruffling, specifically at sites where the loss of cell-cell contact is induced. In addition, antibody-mediated loss of cell-cell adhesion is preceded by and mediated by the production of reactive oxygen species (ROS). Rac1 activity appears to be required for both the antibody-induced disruption of VE-cadherin-mediated cell-cell contacts and the subsequent formation of new junctions. Together, these data show that loss of VE-cadherin function represents a trigger in itself that initiates intracellular signaling that drives the reformation of cell-cell junctions.

**Materials and Methods**

*Reagents and Abs.* Monoclonal antibodies (mAbs) to VE-cadherin (cI75), β-catenin, α-catenin and phosphotyrosine (PY-20) were from Transduction Laboratories (Becton Dickinson Company, Amsterdam, The Netherlands). VE-cadherin mAb 7H1 was from Pharmingen (San Diego, CA, USA). α-Catenin and β-catenin polyclonal Abs were obtained from Santa Cruz (Santa Cruz, CA, USA). The polyclonal Ab to phosphotyrosine was from Zymed Laboratories (Uden, The Netherlands). Recombinant Tumor-Necrosis-Factor (TNF)-α was from PeproTech (Rocky Hill, NJ, USA); Calcein-acetoxymethyl (-AM), Texas-Red Phalloidin, FITC-Dextran 3000, ALEXA-488-labeled Goat-α-Mouse (GoM)-Ig, ALEXA-568-labeled GoM-Ig and ALEXA-488-labeled GoR-Ig secondary
Abs were from Molecular Probes (Leiden, The Netherlands). HRP-labeled Goat-M-Ig or Goat-α-Rabbit (GαR)-Ig was from DAKO (Glostrup, Denmark). Fibronectin (FN) was obtained from the CLB (Amsterdam, The Netherlands). Fetal Calf Serum (FCS) was from Gibco-BRL (Life Technologies, Paisley, Scotland, UK). Basic fibroblast-growth-factor (bFGF) was from Boehringer Mannheim (Mannheim, Germany). Anti-VE-cadherin mAb TEA1.31 was purchased from Immunotech (Marseille, France). EDTA and EGTA were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

**Cell cultures.** Immortalized human umbilical vein endothelial cells (HUVEC) or primary (p)HUVEC, isolated from umbilical cord, were cultured in FN-coated culture flasks (NUNC, Life Technologies) in Medium 199 (Gibco-BRL), supplemented with 20% (v/v) pooled, heat-inactivated FCS, 1 ng/ml bFGF, 5 U/ml heparin, 300 μg/ml glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. After reaching confluency, the endothelial cells were passaged by treatment with trypsin/EDTA (Gibco BRL). In some experiments, endothelial cells were pretreated with 10 ng/ml TNF-α overnight as indicated. All cell lines were cultured at 37°C at 5% CO2.

**Green-Fluorescent-Protein (GFP)-Rac expressing endothelial cells.** The full-length cDNA encoding Rac1 was a kind gift of Dr. J.G. Collard (Netherlands Cancer Institute, Amsterdam, The Netherlands). The Rac1 cDNA was subcloned as a Xho-SnaBI PCR-fragment and swapped with the actin cDNA from the modified LZRS-GFP-actin-IRES-zeocin vector [30]. The resulting construct, LZRS-GFP-Rac-IRES-zeocin, was transfected into amphotropic Phoenix packaging cells [10] by means of the calcium-phosphate transfection system (Life Technologies) to produce retroviruses. HUVECs were transduced with virus-containing supernatant in the presence of 10 μg/ml DOTAP (Boehringer). After 6 hours, the supernatant was replaced with fresh medium, and the cells were allowed to recover overnight. This procedure was repeated twice on two consecutive days. Transduced cells were sorted for GFP expression by FACStar (Becton Dickinson, Heidelberg, Germany).

**Peptide synthesis.** The Rac17-32 peptide, which inhibits Rac1 function [28], was designed in combination with the protein transduction domain of the HIV Tat-protein
The resulting peptide (YGRKKRRQRRRGTCLLISYTTNAFPGEY) was synthesized at the Netherlands Cancer Institute, Amsterdam, The Netherlands.

Immunocytochemistry. HUVEC were cultured on FN-coated glass cover slips, fixed and immunostained as described [8] with a mAb to VE-cadherin (7H1, 10 µg/ml) or to phosphotyrosine (PY-20, 10 µg/ml). Polyclonal anti-phosphotyrosine (10 µg/ml), anti-α-catenin (10 µg/ml) and anti-β-catenin (10 µg/ml) were used when endothelial cells were pretreated with mAbs to VE-cadherin (25 µg/ml). Subsequent visualization was performed with fluorescently-labeled secondary Abs (10 µg/ml). F-actin was visualized with Texas-Red Phalloidin (1 U/ml). In some experiments, cells were pretreated for 30 minutes at 37°C with 20 µg/ml Rac17-32 peptide, followed by washing. Images were recorded with a ZEISS LSM510 confocal microscope with appropriate filter settings. Cross-talk between the green and red channel was avoided by use of sequential scanning.

Electric Cell-substrate Impedance Sensing (ECIS). Endothelial cells were seeded at 100,000 cells per well (0.8 cm²) on FN-coated electrode arrays and grown to confluency. After the electrode check of the array and when the basal electrical resistance of the endothelial monolayer reached a plateau, Abs to VE-cadherin were added and electrical resistance was monitored on-line at 37°C at 5% CO₂ with the ECIS-Model-100 Controller from BioPhysics, Inc. (Troy, NY, USA). After eight hours, data were collected and changes in resistance of endothelial monolayer were analyzed.

Immunoprecipitation and Western blot analysis. Cells were grown to confluency on FN-coated dishes (50 cm²), washed twice gently with ice-cold Ca²⁺- and Mg²⁺-containing PBS and lysed in 1 ml of lysis buffer (25 mM Tris, 150 mM NaCl, 10 mM MgCl₂, 2 mM EDTA, 0.02% (w/v) SDS, 0.2% (w/v) deoxycholate, 1% NP-40, 0.5 mM orthovanadate with the addition of fresh protease-inhibitor-cocktail tablets (Boehringer Mannheim) pH 7.4). After 10 minutes on ice, cell lysates were collected and precleared for 30 minutes at 4°C with protein-G Sepharose (Pharmacia Biotech, Uppsala, Sweden, 15 µl for each sample). The supernatant, separated by centrifugation (14,000g, 15 seconds at 4°C) was incubated with 15 µl of protein-G Sepharose that had been coated with 5 µg/ml β-catenin mAb for 1 hour at 4°C under continuous mixing. The beads were washed 3 times in lysis buffer and proteins were eluted by boiling in SDS-sample buffer.
containing 4% 2-mercaptoethanol (Bio-Rad). The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to 0.45-μm nitrocellulose (Schleicher and Schnell Inc., NH, USA) and the blots were blocked with blocking buffer (1% (w/v) low-fat milk in TBST) for 1 hour, subsequently incubated at room temperature with the appropriate Abs for 1 hr, followed by incubation with RαM-Ig-HRP for 1 hr at room temperature. Between the various incubation steps, the blots were washed 3 times with TBST and finally developed with an enhanced chemiluminescence (ECL) detection system (Amersham).

**Racl and RhoA activity assays.** Cells were stimulated for the indicated times with cl75 (25 μg/ml) or 5 mM EDTA and put on ice. Cells were washed with ice-cold PBS, lysed for 10 minutes on ice in lysis buffer and assayed for Rac activation, as described by Sander and co-workers [23]. The RhoA activity assay was carried out in the same way as the Rac1 activity assay, with GST-Rhotekin instead of GST-PAK. Finally, in both assays, the beads were washed four times with lysis buffer; the fourth time, the beads were put in new tubes and subsequently suspended in 2x-sample buffer containing 4% 2-mercaptoethanol. Samples were analyzed by SDS-PAGE as described above.

**Cell fractionation.** Cells were grown to confluency on FN-coated dishes (50 cm²), washed twice gently with ice-cold Ca²⁺- and Mg²⁺-containing PBS and lysed in 1 ml of lysis buffer, as described above, containing 1% Triton-X-100 instead of 1% NP-40. After 10 minutes on ice, cell lysates were collected and separated by centrifugation (14,000g, 1 minute at 4°C). The pellet fraction contained Triton-X-100-insoluble proteins, associated to the actin cytoskeleton, and the supernatant contained Triton-X-100-soluble, cytosolic proteins. Samples were boiled in SDS-sample buffer containing 4% 2-mercaptoethanol (Bio-Rad), and were immediately analyzed by SDS-PAGE and continued as described above.

**Measurement of reactive oxygen species (ROS).** To measure generation of reactive oxygen species (ROS) in endothelial cells, pHUVECs cultured on fibronectin-coated glass coverslips were loaded with dihydrodoramine-1,2,3 (DHR, 30 μM; Molecular Probes) for 30 min, washed and subsequently treated with the VE-cadherin Ab cl75, control Ab IgG, or medium. Fluorescence of DHR was quantitated by time-lapse

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confocal microscopy. Intensity values are shown as the percentage increase relative to the basal DHR values at the start of the experiment.

Results

VE-cadherin is an essential junctional protein that specifically localizes to adherens junctions and controls endothelial integrity. Using antibodies (Abs) that recognize distinct epitopes in the extracellular domain of VE-cadherin, we and others have described differential effects of these Abs on the permeability of endothelial cells [3;4;6;25]. However, these studies were in part based on diffusion of a fluorescently labeled high-molecular weight marker over the endothelial monolayer to measure the endothelial integrity. In our initial experiments, we used a more sensitive approach, which is based on real-time analysis of the electrical resistance of endothelial monolayers. This analysis shows that the VE-cadherin-blocking Ab c175 rapidly reduces the resistance of the endothelial monolayer, whereas TEA1.31 did so only partially (Figure 1A). The non-blocking Ab 7H1 did not have any effect on the resistance of the endothelial cells (Figure 1A). Previous reports from our group and from others have shown that the VE-cadherin- Abs are able to disrupt endothelial junctions and induce a redistribution of VE-cadherin over the endothelial-cell surface [3;8;25]. We now found that also α- and β-catenin became diffusely distributed, when the endothelial cells were treated with the c175 Ab (Figure 1B).

In sparse endothelial-cell cultures, the tyrosine phosphorylation levels of the VE-cadherin complex are relatively high [12]. Since c175 induces loss of endothelial cell-cell contacts, we studied the effects of c175 on the tyrosine-phosphorylation levels of junctional proteins in confluent monolayers. Immunocytochemical analysis showed that in control situations, most tyrosine-phosphorylated proteins appear to reside in focal adhesions (Figure 2A). A brief exposure to c175 induced increased tyrosine phosphorylation at endothelial cell-cell junctions (Figure 2A). To establish that these results were due to loss of VE-cadherin-mediated cell-cell contacts and not to an unspecific effect of the Ab, we induced junctional disruption with EGTA. EGTA sequesters extracellular calcium and blocks VE-cadherin-mediated, calcium-dependent
cell-cell contacts, similar to cl75. EGTA treatment also increased phosphotyrosine levels at endothelial junctions (data not shown).

Figure 1. Blocking antibodies to VE-cadherin decrease endothelial electrical resistance (A) Modulation of HUVEC monolayer electrical resistance was analyzed as described in Methods. At the start of the experiment, antibodies to VE-cadherin (25 μg/ml) were added (open arrowhead). The control anti-VE-cadherin Ab 7H1 did not affect the electrical resistance (filled line), whereas the TEA1.31 (dashed line) and cl75 (dotted line) Abs rapidly decreased the electrical resistance of the endothelial monolayers. Experiments were performed at least five times in duplicate. A representative experiment is shown. (B) Endothelial cells were cultured and grown to confluency on FN-coated glass cover slips and subsequently incubated with 7H1 antibody (a,b,c,d) or cl75 (e,f,g,h) for 30 minutes. Incubation of the cells with cl75 induced loss of cell-cell contacts and redistribution of β-catenin and α-catenin over the endothelial cell-surface. β-Catenin is shown in green (a,e), F-actin in red (b,f) and yellow shows co-localization between β-Catenin and F-actin (c,g). α-Catenin is in green (d,h). Nuclear staining is due to a-specific binding of the polyclonal antibody. Bars, 20 μm.
Biochemical analysis of β-catenin immunoprecipitates showed that cl75 primarily induced the tyrosine phosphorylation of β-catenin (Figure 2B). Additional analysis revealed that cl75-induced loss of cell-cell contact did not result in the dissociation of VE-cadherin from β-catenin (Figure 2B). Surprisingly, the levels of VE-cadherin-associated α-catenin increased upon cl75-treatment, whereas those of β-catenin did not or only slightly (Figure 2C). Since the association between β-catenin and VE-cadherin was hardly altered upon loss of VE-cadherin-mediated cell-cell contacts, we used Abs to β-catenin to immunoprecipitate the complex and showed that both cl75 and EDTA promoted the association of α-catenin to the VE-cadherin complex (Figure 2D). These findings show that loss of VE-cadherin function induces increased tyrosine phosphorylation of β-catenin and also increases the association of α-catenin to β-catenin present in the VE-cadherin complex.

To study whether loss of VE-cadherin function and, as a consequence, loss of VE-cadherin-mediated cell-cell contacts, induced dissociation of α-catenin from the actin cytoskeleton, we fractionated the cl75-treated endothelial cells and analyzed the cytoskeletal and the cytosol/membrane fraction for the presence of α-catenin. These experiments showed that α-catenin dissociated from the cytoskeleton when VE-cadherin-mediated cell-cell contacts were disrupted by either cl75 or EDTA treatment (Figure 3). Also β-catenin translocated to the same fraction as α-catenin upon loss of cell-cell contacts (data not shown), indicating that the entire VE-cadherin complex becomes dissociated from the cytoskeleton.

Real-time analysis by phase-contrast microscopy revealed that endothelial cells rapidly lost contact and contracted upon exposure to the cl75 antibody, followed by the induction of membrane ruffles (Figure 4A; see for video Quick-Time movie Fig4A.mov). Similarly, EGTA treatment also resulted in a rapid loss of cell-cell contacts, which was followed by induction of membrane ruffles. Intriguingly, the ruffles appeared specifically at sites where the endothelial cells had just lost contact with the neighboring cell (Figure 4B; see for video Quick-Time movie Fig4B.mov).
Figure 2. Antibody-mediated loss of cell-cell contacts induces tyrosine phosphorylation of junctional proteins. (A) Endothelial cells were cultured and grown to confluency on FN-coated glass cover slips and
treated with 7H1 Ab (a,b,c) or cl75 (d,e,f) for 15 minutes. Cells were fixed, permeabilized and stained for phosphotyrosine in green (pY; a,d) and F-actin in red (b,e). Merged images are shown in panels c and f. Ab cl75 increased the levels of phosphotyrosine, in particular at the cell-cell junctions. Bars, 50 μm. (B) Cells were cultured as described in Methods, and treated for 30 minutes with cl75, followed by β-catenin immunoprecipitation (I.P.). Samples were analyzed by SDS-PAGE and subsequently analyzed by immunoblot (I.B.) with specific mAbs to phosphotyrosine (pY), VE-cadherin and β-catenin. Upper panel shows increased phosphotyrosine levels of β-catenin after 30 minutes of cl75-induced loss of cell-cell contacts in primary HUVEC. The middle panel shows that VE-cadherin and β-catenin remain associated; the I.P. for β-catenin and I.B. for VE-cadherin (7H1) show no changes in the level of their association. Lower panel shows equal amounts of β-catenin in the I.P. (C) I.P. of VE-cadherin (TEA1.31) shows that α-catenin becomes enriched in the VE-cadherin I.P. (upper panel) and that the levels of associated β-catenin remain unaltered (lower panel) upon treatment with cl75. Note that the cl75 Ab will not immunoprecipitate VE-cadherin in the lysis buffer used, due to a lack of calcium. (D) I.P. of β-catenin shows that more α-catenin associates with β-catenin upon EDTA (7 minutes)-induced loss of VE-cadherin-mediated cell-cell contacts (upper panel). Lower panel represents the loading control for β-catenin. All experiments were repeated at least two times.

Figure 3. α-Catenin dissociates from the actin cytoskeleton upon loss of VE-cadherin-mediated cell-cell adhesion. Cells were cultured as described in Methods and treated as indicated for 30 minutes, except for EDTA (7 minutes). Cells were lysed in Triton-X-100 buffer and fractions were separated in a cytosolic and membrane fraction (defined as Triton-X-100 soluble) and a cytoskeletal fraction (Triton-X-100 insoluble) and analyzed by SDS-PAGE. Immunoblots (I.B.) were probed for α-catenin and show that cl75 and EDTA treatment induce a shift of α-catenin from the cytoskeletal fraction to the cytosolic and membrane fraction. A representative experiment from three independent experiments is shown.
Detailed real-time analysis of a particular endothelial junction showed that its dissociation was immediately followed by the induction of membrane ruffles from both endothelial cells, exactly at the site where the cells had dissociated (Figure 4C; see for video Quick-Time movie with filename Fig4C.mov). Moreover, the membrane ruffles appeared dorsal to the retracting membrane. These observations indicate that re-closure of the junction rapidly follows loss of cell-cell adhesion and is initiated by the induction of membrane ruffles at specific sites along the cell-periphery.

Figure 4. Loss of cell-cell junctions induces formation of membrane ruffles and spreading. (A) Cells were plated on glass cover slips and followed in real-time by phase-contrast microscopy. Still phase-contrast images were taken from time-lapse recordings at the times indicated in upper-left corner. At time 0 seconds, cl75 was added (a). After 862 seconds the cells were rounded (b) and started to induce membrane ruffles, as indicated by the open arrowhead (1724 seconds, c). See for video Quick-Time movie Fig4A.mov. Bar, 50 μm. (B) Endothelial cells treated with 5 mM EGTA at time 0 seconds (a) and monitored on-line by confocal microscopy. EGTA rapidly induces loss of cell-cell contacts (asterisk, 884 seconds, b) which is followed by membrane ruffles at sites where the cells just lost contact (open arrowhead, 1863 seconds, c). Still phase-contrast images were taken from time-lapse recording at the times indicated in upper-left corner. See for video Quick-Time movie Fig4B.mov. The experiment was repeated five times. Bar, 50 μm. (C) Detailed analysis of an endothelial cell-cell junction following loss of cell-cell contact (0 seconds, a). Spontaneous loss of cell-cell contact (asterisk, 950 seconds, b) is immediately followed by membrane ruffling (open arrowhead, 1357 seconds, c), until the cell-cell junction is restored (open arrowhead, 2036 seconds, d). Note that the membrane ruffles from both cells appear right at the sites where the cells lost
contact; however, the localization of the ruffle is dorsal to the retracting membrane. Bar, 10 μm. See for video Quick-Time movie Fig4C.mov.

Previously, we have shown by protein transduction that an active mutant of the small GTPase Rac1 induced loss of cell-cell contacts of confluent endothelial cells, followed by membrane ruffling [26]. Thus, the observed phenotype is likely induced by Rac1, a known regulator of membrane ruffles [22]. To test this, we investigated whether loss of VE-cadherin function activated Rac1 by pull-down assays with GST-p21-activating kinase (PAK) and showed that cl75 indeed induces Rac1 activation (Figure 5A). The response was highest at 5 minutes, after which it declined somewhat, although the levels of activation of Rac1 at 30 minutes remained elevated compared to the control samples (Figure 5A). To show that the Rac1 activation was due to the loss of VE-cadherin function, we treated the cells with EDTA. This resulted also in a rapid activation of Rac1 in endothelial cells (Figure 5B).

The fact that the cells respond initially with contraction following loss of VE-cadherin function induced either by the antibody cl75 or by EDTA, was suggestive for activation of RhoA, since RhoA is known to induce cell contraction and stress fiber formation. As expected, RhoA was activated, albeit modestly, with a maximum at 5 minutes, after which the activation levels dropped below control levels at 15 minutes (Figure 5C). These results show that primarily Rac1 and RhoA are activated following the loss of VE-cadherin function.

Antibodies to the extracellular domain of VE-cadherin, such as cl75, inhibit its function, reflected by a reduction of the endothelial monolayer resistance (Figure 1A). Moreover, these antibodies are capable of activating Rac1 (Figure 5A). To define whether the response to the loss of VE-cadherin function depends on Rac1 activity, we used a cell-permeable peptide-inhibitor of Rac1, Tat-Rac17-32. This peptide represents part of the effector loop of Rac1 and competes in cells with Rac1-effector interactions, thus preventing downstream signaling [27;28]. After 30 minutes, cl75 had maximally reduced the endothelial monolayer resistance as is shown in figure 1A and this reduction was set to 100%.
Figure 5. Rac1 is rapidly activated after loss of VE-cadherin-mediated cell-cell contacts. (A) Cells were cultured and treated as indicated and described in Methods. Rac1 activity assay (upper panel) shows that Rac1 was activated after 5 minutes of cl75-treatment (Rac.GTP). Lower panel shows equal protein loading (Total Rac). (B) Cells were treated as in A, with EDTA for 5 minutes to dissociate VE-cadherin-mediated cell-cell contacts. EDTA treatment induced activation of Rac1 (upper panel, Rac.GTP). Lower panel shows equal protein loading (Total Rac). (C) Cells were treated as in A. RhoA pull-down assay (upper panel) shows that RhoA was activated after 1 minute of cl75 treatment (RhoA.GTP). Lower panel shows equal protein loading (Total RhoA). Experiments were done at least two times.

The Tat-Rac17-32-incubated monolayers showed a reduced response to the cl75 treatment (approximately 50% reduction in resistance, Figure 6A). This indicates that the loss of VE-cadherin function, induced by cl75, depends at least in part on the activity of Rac1. Additionally, we studied the involvement of PI-3K, a known mediator of Rac1 activation [23], by using the LY-294002 compound. Similar to the inhibitor of Rac1,
inhibition of PI-3K had only a partial effect (approximately 25%) on the cl75-induced decrease of the endothelial monolayer resistance (data not shown). We previously showed that Rac1 activation in endothelial cells induces a RhoA phenotype, i.e. stress fibers and cell-contraction [26]. Therefore, we tested an inhibitor of Rho-kinase, a downstream effector of RhoA, to assess the role for RhoA in VE-cadherin function. The Rho-kinase inhibitor Y-27632 did prevent the cl75-mediated reduction of the electrical resistance of the monolayer, although not as effective as the Rac1 inhibitor (Figure 6A). Previous work had already indicated that also the RhoA-inhibiting C3 transferase had a minor effect on cl75-induced reduction of the monolayer resistance [25].

Interestingly, under control conditions, we observed a small decrease in the electrical resistance of the endothelial monolayer when the confluent endothelial monolayers were incubated with Tat-Rac1-32 alone or with the PI-3K inhibitor alone (Figure 6A and data not shown). This suggests that in order to maintain the endothelial junctions, a low level of active Rac1 is required. In conclusion, these findings suggest that the loss of cell-cell contact, induced by cl75, requires active Rac1 and, to a limited extent, PI-3K and Rho-kinase.

Previous work had revealed that active Rac1 mediates the loss of endothelial cell-cell adhesion through production of reactive oxygen species (ROS) [26]. Scavenging ROS, by treating the cells with N-acetylcysteine (N-AC), resulted in inhibition of cl75-induced loss of cell-cell contacts (Figure 6B). In line with this result, the cl75-induced drop in endothelial monolayer resistance was prevented by N-AC (data not shown). These findings indicate that production of ROS is required to mediate the loss of VE-cadherin-mediated cell-cell adhesion. Consistently, cl75-induced loss of VE-cadherin-mediated cell-cell contacts promoted a rapid increase in ROS production. (Figure 6C; see for video Quick-Time movie Fig6DHR.mov). Additional experiments showed that irrelevant antibodies such as isotype control IgG1 or medium changes do not mimic the induction of ROS. Moreover, the ROS production is rapidly followed by loss of cell-cell contacts, as is observed with phase-contrast real-time microscopy imaging (Figure 6D; see for video Quick-Time movie Fig6Phase.mov). Since ROS is found to act downstream of Rac1 activity, these observations indicate that ROS play an important role in the control of the dynamics of VE-cadherin-mediated cell-cell adhesion.
Figure 6. VE-cadherin-mediated loss of cell-cell contacts requires Rac1 activity and ROS. (A) Cells were treated for 30 minutes with either the Tat-Rac17-32 peptide, with the Rho-kinase inhibitor Y-27632 or left untreated and further processed as described in Methods and legends of Figure 1A. The cl75-induced decrease in resistance at 30 minutes is set to 100% (filled bars). The reduction in resistance by cl75 of the Tat-Rac17-32-treated monolayers was less compared to the control (inhibition by the Tat-Rac17-32 peptide of 52%). The cl75-induced resistance of the Y-27632-treated monolayers was inhibited for 37%. Spontaneous resistance of the Tat-Rac17-32-treated monolayers was decreased by 24% (open bars). Data represent three experiments, performed in duplicate. (B) Endothelial cells were cultured and grown to confluency on FN-coated glass cover slips and pretreated with 5 mM N-AC overnight or left untreated. Subsequently, the cells were incubated with cl75 (a-f) for 30 minutes. Scavenging of ROS prevented cl75-induced loss of cell-cell contacts. VE-cadherin is shown in green (a,d), F-actin in red (b,e) and yellow shows co-localization between VE-cadherin and F-actin (c,f). A representative experiment, performed twice, is shown. Bar, 50 μm. (C) Endothelial cells were cultured and grown to confluency on FN-coated glass cover slips and incubated with DHR, as described in Methods. Time-lapse recordings of increases in fluorescence are measured by confocal microscopy and reflect increased ROS production. Value at time 0 represents the basal presence of ROS in resting endothelial cells. Closed circles represent ROS production in endothelial cells after cl75 treatment, i.e. loss of VE-cadherin function, open circles represent ROS production in endothelial cells after addition of medium. Experiment is done twice in duplicate. (D) Endothelial cells were cultured and grown to confluency on FN-coated glass cover slips, incubated with
DHR, as described in Methods, and treated with cl75 to induce loss of cell-cell contacts. Upper panels are confocal images, taken from time-lapse recordings and show increase in DHR signal in endothelial cells. Time in seconds is indicated in upper-left corner. Lower panels are the phase-contrast images, taken from same time-lapse recordings as presented in upper panels and show morphological changes of the endothelial monolayer upon cl75 addition. Experiment was done twice in duplicate. Bar, 100 μm. Videos of this experiment are included as Quick Time movies. Movie 1 upper panel: Fig6DHR.mov; movie 2 lower panel: Fig6Phase.mov).

Transduction of GFP-Rac into endothelial cells revealed that Rac1 was not only localized in membrane ruffles (Figure 7A, see for video Quick-Time movies Fig7AGFP.mov and Fig7APhase.mov), but also at endothelial cell-cell junctions, where it co-localized with VE-cadherin (Figure 7B). In addition, stable endothelial junctions still showed GFP-Rac expression. Real-time analysis of the formation of newly formed cell-cell contacts with GFP-Rac-transduced endothelial cells showed that GFP-Rac stably localized at the newly formed endothelial cell-cell junction (Figure 7C; see for video Quick-Time movies Fig7CGFP.mov and Fig7CPhase.mov), suggesting that Rac1 activity may be required for the establishment of new cell-cell contacts. To test this, we first decreased the monolayer resistance by adding EGTA to the endothelial cells and at the moment when the lowest resistance was measured, we added fresh medium plus the Rac17-32 peptide and studied the recovery of the endothelial monolayer resistance in time (Figure 7D). Control monolayers showed a recovery of their electrical resistance within 2 hours, whereas the Rac17-32 treated monolayers were unable to restore the resistance.

The presented data suggest that VE-cadherin-mediated cell-cell adhesions require a basal level of Rac1 activity to maintain their integrity and barrier function. Changes in VE-cadherin-mediated cell-cell adhesions rapidly elevate Rac1 activity, first to induce ROS production and secondly to restore the barrier function of the endothelial monolayer.

Discussion

This study focuses on the effects of the loss of VE-cadherin-function induced by a specific VE-cadherin antibody and describes a novel and specific response of endothelial
cells to loss of integrity that drives subsequent rapid restoration of VE-cadherin-mediated cell-cell adhesion.

Figure 7. Rac-GFP is localized to ruffles and endothelial cell-cell junctions. (A) GFP-Rac (green) localizes in membrane ruffles (open arrowheads, a). See for time lapse recordings Fig7AGFP.mov and Fig7APhase.mov. Bar, 20 μm. (B) GFP-Rac-transduced endothelial cells were cultured on FN-coated glass cover slips, fixed, permeabilized and analyzed with CLSM. Green staining represents GFP-Rac (a), VE-cadherin appears in red (b) and yellow indicates co-localization (c, merge). Bar in c, 50 μm. Zoomed image in d represents magnification of the square in c. Open arrowheads show GFP-Rac co-localization with VE-cadherin at the cell-cell junctions. Bar, 10 μm. (C) Endothelial cells were transduced with GFP-Rac and seeded on FN-coated glass cover slips and monitored by confocal microscopy. Still images are taken from time-lapse recordings and show GFP-Rac in green (a,b,c). The phase-contrast images are shown in panels d,e and f. GFP-Rac was targeted to new cell-cell contacts (open arrowhead in a,b,c) and once the cell-cell contact is established (open arrowhead), Rac-GFP remained localized to these sites. Time in seconds is shown in upper-left corner. Bar, 10 μm. See for videos Quick-Time movies Fig7CGFP.mov and Fig7CPhase.mov. (D) Endothelial cells were cultured until confluency for analysis by ECIS, as described in Methods. At the start of the experiment, EGTA (triangles or solid line) or medium (filled circles) was added to the well, as indicated by the asterisk. Open arrow-head shows the maximum drop in resistance,
induced by EGTA and replacement of EGTA medium for calcium-rich medium without (triangle line) or with Tat-Rac17-32 peptide (solid line). Inhibition of downstream effectors of Rac1 by Tat-Rac17-32 peptide prevents the recovery of the endothelial monolayer resistance, i.e. the reformation of cell-cell contacts. Representative experiment is shown. Experiments were performed at least two times in duplicate.

Cadherin-induced signaling has recently received much interest. Several reports describe the use of cadherin engagement to induce intracellular signaling, thereby mimicking the formation of cadherin-based junctions. These studies showed that E-cadherin engagement promotes Rac1 activation [11;18]. Interestingly, endothelial and epithelial cell-cell junctions are regulated differently by Rac1, as has been observed by our group as well as by others [2;9;26;31]. Rac1 promotes strong cell-cell adhesion in epithelial cells, but it reduces cell-cell adhesion in endothelial cells, although membrane ruffles and cell spreading require Rac1 activity in both cell types. Whereas several studies have described the effect of active Rac1 on cell-cell junctions by introducing Rac1 mutants into the cells, in the present paper we show that the initial loss of VE-cadherin-mediated cell-cell contacts induces activation of endogenous Rac1, as well as of RhoA. In contrast to the transient RhoA activation, Rac1 activation is prolonged, which corroborates our findings that active Rac1 is not only required for the loss of VE-cadherin-based cell-cell contacts, but is also involved in the re-formation of cell-cell contacts.

Nimnual and colleagues have recently shown that RhoA inhibition through Rac1 depends on reactive oxygen species (ROS) and involves inhibition of the low-molecular-weight protein tyrosine phosphatase (LMW-PTP) [17]. Our findings, which implicate ROS in the loss of VE-cadherin-based cell-cell adhesion, support the idea that RhoA activity is decreased by Rac1-mediated ROS production. Additionally, in line with the notion that H2O2, as a product of ROS, oxidizes critical cysteine residues thereby deactivating tyrosine phosphatases, we found that loss of VE-cadherin function decreased the phosphotyrosine levels of the β-catenin-interacting phosphatase SHP-2 (JDvB, unpublished results). In addition, scavenging ROS decreased the basal phosphotyrosine levels at endothelial cell-cell junctions (JDvB, unpublished results). These results indicate that Rac1-mediated production of ROS is essential for phosphorylation events at cell-cell junctions that occur upon inhibition of VE-cadherin function. Thus, VE-cadherin-
mediated loss of cell-cell adhesion requires concerted action of RhoA- and Rac1-mediated signaling and involves ROS, whereas proper reformation of VE-cadherin-based cell-cell junctions depends mainly on Rac1 activity.

The loss of VE-cadherin-mediated cell-cell adhesion was found to be accompanied by increased tyrosine phosphorylation of β-catenin. This is in line with data from Lampugnani and co-workers, who have shown elevated phosphotyrosine levels of the VE-cadherin complex in sub-confluent endothelial monolayers [12]. Similarly, Ozawa and Kemler reported that pervanadate treatment, leading to tyrosine kinase activation and phosphorylation of junctional proteins, results in loss of E-cadherin function and cell-cell contacts [19]. These authors proposed that the dissociation of the cadherin complex from the actin cytoskeleton underlies the dysfunction of E-cadherin. In agreement with this notion, we found that loss of VE-cadherin function induces the dissociation of the VE-cadherin-catenin complex from the actin cytoskeleton. These results support the idea that tyrosine phosphorylation of β-catenin and the interaction of the VE-cadherin-catenin complex with the actin cytoskeleton critically determine the strength of VE-cadherin-mediated cell-cell adhesion [21].

Hirano and colleagues have shown that α-catenin is crucial for strong cadherin-based adhesion [7]. In addition, it has been suggested that increased tyrosine phosphorylation levels of β-catenin might trigger the dissociation of α-catenin from β-catenin [20]. Surprisingly, we found that α-catenin increased its association to β-catenin upon loss of VE-cadherin-based cell-cell contacts. The increased association is observed after the VE-cadherin antibody-induced drop in endothelial monolayer resistance, when the tyrosine phosphorylation levels of β-catenin are still elevated. However, Piedra and co-workers showed that induced phosphorylation of Tyr-142 β-catenin by Fer or Fyn tyrosine kinases resulted in reduced association of β-catenin to α-catenin in epithelial cells [20]. Interestingly, recent data from the same group showed that tyrosine kinase Fer phosphorylates plakoglobin on Tyr549 and thereby increases its binding to α-catenin [13]. In our study, tyrosine phosphorylation in endothelial cells occurs after the loss of VE-cadherin function. Until now, it is not known which tyrosine residues of β-catenin are phosphorylated in either event or which tyrosine kinase is involved, but it is clear that phosphorylated junctional proteins play an important role in the regulation of cell-to-cell
junctions. We hypothesize that the increased association of \( \alpha \)-catenin to \( \beta \)-catenin following the loss of cell-cell contact might be important to ensure efficient restoration of VE-cadherin-based cell-cell junctions by increasing the interaction of the VE-cadherin-catenin complex to the actin cytoskeleton. However, future experiments should be performed to study this latter hypothesis in detail.

Although it is clear from the present study that Rac1 mediates both the loss and the restoration of VE-cadherin-based cell-cell adhesion, the location of Rac1 activity may be different. The data suggest that the interaction of Rac1 with downstream targets, its intracellular targeting and thus the outcome of Rac1-mediated signaling is determined by the adhesive status, and perhaps localization, of VE-cadherin. Although we tried to implicate the Rac guanosine-nucleotide-exchange factors Vav1, Vav2 and Tiam1, we were unable to show involvement of these molecules in VE-cadherin-mediated loss of cell-cell contacts. Further analysis of the pathway that leads to VE-cadherin-dependent modulation of Rac1-signaling represents an important goal for future studies.

In conclusion, this paper presents new information about the molecular events that accompany endothelial damage and that drive the subsequent repair response. It appears that VE-cadherin is of key importance in this process. Loss of cell-cell adhesion through inhibition of VE-cadherin results in activation of signaling, which leads to cell retraction followed by spreading and restoration of cell-cell adhesion, as is illustrated in figure 8. The current data on how endothelial cells regulate their cell-cell junctions in order to maintain proper barrier function is important to better understand a wide range of (patho) physiological processes related to vascular integrity, such as leukocyte extravasation, angiogenesis, ischemia-reperfusion injury and chronic inflammatory disorders.

References


