

Vascular-endothelial-cadherin modulates endothelial monolayer permeability

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SUMMARY

Vascular endothelial (VE)-cadherin is the endothelium-specific member of the cadherin family of homotypic cell adhesion molecules. VE-cadherin, but not the cell adhesion molecule platelet/endothelial cell adhesion molecule (PECAM-1), markedly colocalizes with actin stress fibers at cell-cell junctions between human umbilical vein endothelial cells. Inhibition of VE-cadherin-mediated, but not PECAM-1-mediated, adhesion induced reorganization of the actin cytoskeleton, loss of junctional VE-cadherin staining and loss of cell-cell adhesion. In functional assays, inhibition of VE-cadherin caused increased monolayer permeability and enhanced neutrophil transendothelial migration.

In a complementary set of experiments, modulation of the actin cytoskeleton was found to strongly affect VE-cadherin distribution. Brief stimulation of the β 2-

adrenergic receptor with isoproterenol induced a loss of actin stress fibers resulting in a linear, rather than 'jagged', VE-cadherin distribution. The concomitant, isoproterenol-induced, reduction in monolayer permeability was alleviated by a VE-cadherin-blocking antibody. Finally, cytoskeletal reorganization resulting from the inactivation of p21Rho caused a diffuse localization of VE-cadherin, which was accompanied by reduced cell-cell adhesion. Together, these data show that monolayer permeability and neutrophil transendothelial migration are modulated by VE-cadherin-mediated cell-cell adhesion, which is in turn controlled by the dynamics of the actin cytoskeleton.

Key words: VE-cadherin, Cytoskeleton, Permeability, Transmigration

INTRODUCTION

Homotypic cell-cell adhesion between epithelial or endothelial cells is mediated by adhesion structures such as adherens junctions, tight junctions and desmosomes. Members of the cadherin family of calcium-dependent homotypic cell-cell adhesion molecules are largely confined to the adherens junctions (Gumbiner, 1996; Lampugnani and Dejana, 1997). Endothelial cells express both VE- (vascular endothelial) and N- (neuronal) cadherins. Whereas VE-cadherin is localized at sites of cell-cell contact, N-cadherin is distributed diffusely over the cell surface and does not seem to contribute to cell-cell adhesion (Lampugnani and Dejana, 1997).

Similar to other cadherins, the cytoplasmic tail of VE-cadherin associates with the armadillo-family members β -catenin, plakoglobin (γ -catenin) and p120. The connection of the cadherin-catenin complex to the actin cytoskeleton is mediated by α -catenin (Lampugnani et al., 1995; Rimm et al., 1995). Transfection experiments and gene-inactivation studies have shown that VE-cadherin expression reduces monolayer permeability, promotes cell aggregation, motility and growth and that VE-cadherin is required for the organization of vascular-like structures in embryoid bodies

(Navarro et al., 1995; Caveda et al., 1996; Vittet et al., 1997). Recently, a second VE-cadherin was cloned that also localized to intercellular junctions but did not associate with catenins or the cytoskeleton and did not modulate either monolayer permeability, cell migration or growth (Telo' et al., 1998).

The Ig-like cell adhesion molecule CD31/PECAM-1 (platelet/endothelial cell adhesion molecule) is expressed on platelets, leukocytes and endothelial cells (Newman, 1997). PECAM-1 is, like VE-cadherin, concentrated at the sites of endothelial cell-cell contact and mediates cell-cell adhesion between endothelial cells and adherent leukocytes through homotypic as well as heterotypic interactions (Albelda et al., 1991; Newman, 1997). PECAM-1 has been implicated in endothelial tube formation, cell migration and angiogenesis (Matsumura et al., 1997; DeLisser et al., 1997; Kim et al., 1998), and plays an important role in the transendothelial migration of a wide range of leukocytes, including neutrophils, T-lymphocytes, monocytes and CD34⁺ hematopoietic precursor cells (Muller et al., 1993; Vaporciyan et al., 1993; Zocchi et al., 1996; Yong et al., 1998).

Regulation of cadherin-mediated homotypic cell-cell adhesion has extensively been studied in epithelial cells

(Gumbiner, 1996). Cadherin function was recently shown to be regulated by the small GTPases Rho, CDC42 and in particular Rac, which control the dynamics of the actin cytoskeleton. These studies revealed a crucial role for the cortical actin cytoskeleton in the control of E-cadherin-mediated cell-cell adhesion (Braga et al., 1997; Hordijk et al., 1997; Kuroda et al., 1997; Takaishi et al., 1997). To study the role of VE-cadherin and its regulation by the actin cytoskeleton in the control of endothelial cell-cell adhesion, we performed a functional and morphological analysis of the VE-cadherin-cytoskeleton connection in human umbilical vein endothelial cells (HUVEC).

The present study shows that VE-cadherin, but not PECAM-1, preferentially colocalizes with actin stress fibers and that blocking VE-cadherin function leads to a marked reorganization of the actin cytoskeleton, increased monolayer permeability and enhanced neutrophil transmigration. Conversely, remodeling of the actin cytoskeleton significantly altered VE-cadherin, but not PECAM-1, distribution. The results support a model in which monolayer properties are determined by the dynamics of the endothelial actin cytoskeleton through its modulation of VE-cadherin-mediated cell-cell adhesion.

MATERIALS AND METHODS

Antibodies and reagents

Anti-VE-cadherin antibodies were either from Transduction Laboratories (cl75), Pharmingen (55-7H1) or a kind gift from Dr E. Dejana, Milano (TEA1-31). Anti- β - and anti- γ -catenin and anti-phosphotyrosine (PY20) were also from Transduction Laboratories. Antibodies to CD31/PECAM-1 were generated in our own institute and have been described (Van Mourik et al., 1985). Recombinant human interleukin (IL)-8 and IL-1 β were from PreproTech; fMLP and platelet-activating factor (PAF) were from Sigma. Calcein-acetoxymethyl (-AM), Texas Red phalloidin, jasplakinolide, fluorescein isothiocyanate (FITC)-Dextran 3000 and FITC-Dextran 40000 and ALEXA488 goat anti-mouse-Ig secondary antibody all were from Molecular Probes. Phycoerythrin (PE)-labelled secondary antibodies were from DAKO. Human serum, human serum albumin (HSA) and fibronectin were obtained from our own institute (CLB). Foetal calf serum (FCS) was purchased from Gibco. Basic fibroblast growth factor (bFGF) was from Boehringer Mannheim.

Cell culture

HUVEC that were either freshly isolated (Brinkman et al., 1994) or immortalized by the human papilloma virus E6/E7 proteins (Fontijn et al., 1995) were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated human serum, 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine and 5 ng/ml bFGF. All experiments were initially performed with the immortalized HUVEC (4th-15th passage) and the results were confirmed with freshly isolated HUVEC (2nd-4th passage), yielding comparable results. Cells were subcultured either on fibronectin-coated (10 μ g/ml, 30 minutes, 37°C) glass coverslips in 6- or 12-well plates for use in immunocytochemistry or on fibronectin-coated polycarbonate membranes (3.0 μ m pore size, 12 mm diameter) in Transwells (Costar). For studies in Transwells, 150,000 HUVEC (in 0.5 ml of culture medium) were added to the upper compartment, non-adherent cells were removed after 18 hours and monolayers were cultured for 3-5 days. The lower compartment of these Transwells contained 1.5 ml of medium during culture and during the experiments. Cells were pretreated with 10 μ g/ml IL-1 β for 4 hours prior to the experiments.

Granulocyte isolation

Blood was obtained from healthy volunteers. Granulocytes were isolated from a buffy coat of 500 ml of blood by density gradient centrifugation over isotonic Percoll (Pharmacia) (Roos and de Boer, 1986). After lysis of the erythrocytes in the pellet fraction with cold lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA, pH 7.4), the granulocytes were washed twice with PBS and resuspended in Hepes medium (132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl₂, 1.0 mM Mg₂SO₄, 1.2 mM KH₂PO₄, 20 mM Hepes, 5.5 mM glucose and 0.5% (w/v) HAS, pH 7.4, at 10⁷ cells/ml.

Permeability assays

Permeability of HUVEC monolayers cultured on Transwell filters was assayed using FITC-labelled dextrans. Pilot experiments showed that comparable results were obtained when either FITC-dextran 3000 or -40000 was used; results obtained with FITC-dextran 3000 are shown. Permeability experiments were started by washing the upper and lower compartment with Hepes medium, followed by the addition of the FITC-Dextran (10 μ g/ml) in 0.5 ml Hepes medium to the upper compartment. After 2 hours, the medium from the lower compartment was collected and fluorescence was measured in a spectrofluorimeter (λ_{EX} 485 nm; λ_{EM} 525 nm). In some experiments, monolayers were pretreated with antibodies (10 μ g/ml, 1 hour) or with a combination of isoproterenol (10 μ M) and IBMX (3-isobutyl-1-methyl xanthine, 1 mM) for 30 minutes. All reagents were also present during the assays. Data represent mean \pm s.d. from a representative of at least three experiments.

Transmigration assays

Transendothelial migration was measured using endothelial monolayers cultured on 3 μ m pore size polycarbonate filters in Transwells. Prior to the assays, neutrophils (10⁷/ml) were labeled with 4 μ g/ml calcein-AM in Hepes medium for 30 minutes at 37°C. After labeling, the cells were washed twice with and resuspended in Hepes medium (10⁶ cells/ml). In all experiments, the upper compartment of the culture inserts was washed twice with Hepes medium prior to the experiment. The medium in the lower compartment was replaced with prewarmed Hepes medium with or without chemoattractants (fMLP or IL-8, 10⁻⁸ M). Calcein-labelled neutrophils (0.5 \times 10⁶ cells) were placed in the upper compartment, and the Transwells were incubated for 30 minutes in a tissue culture incubator. Subsequently, the cells in the upper and lower compartments and in the filter were separately lysed in PBS supplemented with 0.1% (v/v) Tween-20, 0.2% (w/v) hexadecyl-trimethyl-ammoniumbromide (Sigma), 0.2% (w/v) BSA and 20 mM EDTA. The amount of fluorescence in each of these fractions was measured in a spectrofluorimeter (λ_{EX} 485 nm; λ_{EM} 525 nm). The percentage of cells present in each compartment or on the filter, was calculated as a fraction of the input fluorescence (labeled cells directly lysed in lysis buffer) taken as 100%. In some experiments, monolayers were pretreated for 1 hour with anti-VE-cadherin or anti-PECAM-1 antibodies (10 μ g/ml), with cytochalasin D (2 μ M) for 30 minutes, or with Jasplakinolide (10 μ M) for 10 minutes and subsequently washed twice with Hepes medium. Data represent mean \pm s.d. from a representative of at least three experiments and were statistically analyzed with the Student's *t*-test.

Immunocytochemistry and FACS analysis

Endothelial monolayers were cultured on fibronectin-coated glass coverslips and were fixed and immunostained as described (Hordijk et al., 1997) with either PECAM-1 antibodies (20 μ g/ml), anti-VE-cadherin (25 μ g/ml) or anti- γ -catenin (20 μ g/ml) followed by staining with fluorescently labelled secondary antibodies (20 μ g/ml). F-actin was visualized by Texas-Red phalloidin (1 U/ml). In some experiments, cells were pretreated with anti-VE-cadherin or anti-PECAM-1 antibodies (1 hour, 10 μ g/ml). Images were recorded with a LEICA TCS-NT confocal laser scanning microscope using appropriate filter settings and a magnification of \times 630. Crosstalk

between the green and red fluorescence channel, which could give rise to false-positive colocalization, was avoided by careful selection of the imaging conditions. To underscore colocalization of proteins with actin, some images were taken at a 3-fold zoom setting.

The expression of surface antigens on the HUVEC was measured by indirect immunofluorescence and flow cytometry. Following preincubations, the HUVEC were detached with 1 mM EDTA in calcium-free Hepes medium for 15 minutes at 37°C. After harvesting, the cells were incubated in PBS containing 0.5% BSA the presence of 1 mM calcium with the different mAbs (10 µg/ml) for 30 minutes at 4°C and washed with a 30-fold excess of ice-cold PBS/BSA. The cells were then incubated with PE-conjugated goat anti-mouse Ig for 30 minutes at 4°C and washed. The relative fluorescence intensity was measured with a flow cytometer (FACScan, Becton Dickinson).

RESULTS

VE-cadherin colocalizes with the ends of actin stress fibers

The localization of VE-cadherin and PECAM-1 to the intercellular junctions of endothelial cells is well described (Albelda et al., 1991; Ayalon et al., 1994; Dejana, 1996). Immunofluorescence staining in combination with high-magnification confocal microscopy revealed a differential codistribution of VE-cadherin and PECAM-1 with F-actin at the cellular junctions of endothelial cells (Fig. 1). VE-cadherin was distributed in a striped or 'jagged' fashion, due to the colocalization with the endpoints of stress fibers, terminating at the sites of cell-cell contact (Fig. 1). This staining pattern was observed with three different anti-VE-cadherin antibodies in immortalized as well as freshly isolated endothelial cells. This colocalization with the ends of stress fibers was also seen for β -catenin (not shown) and plakoglobin (γ -catenin; Fig. 1), suggesting that the cadherin-catenin complexes are involved in

connecting stress fibers from adjacent cells. In contrast to VE-cadherin, PECAM-1 did not show this colocalization with stress fibers (Fig. 1), but consistently localized to a broad area between the cells, which represents the overlapping cell-cell contact region. Here, small circular areas devoid of PECAM-1-staining were observed, the nature of which is unknown.

Inhibition of VE-cadherin, but not CD31, induces monolayer permeability and promotes neutrophil transmigration

To explore VE-cadherin function in conjunction with the actin cytoskeleton, we made use of a blocking VE-cadherin antibody (c175) and tested its effects in permeability- and transmigration assays. The c175 antibody, but not an IgG1 isotype control (7H1) that is directed against a different epitope on VE-cadherin, increased the permeability of endothelial monolayers for FITC-dextran by approximately twofold (Fig. 2). In contrast, monoclonal antibodies to PECAM-1 did not alter monolayer permeability.

The role of VE-cadherin in monolayer integrity was further investigated by studying IL-8-induced transendothelial migration of neutrophils. As shown in Fig. 3A, inhibition of VE-cadherin-mediated cell-cell adhesion by the c175 antibody promoted basal and IL-8-induced transendothelial migration of neutrophils. Inhibition of VE-cadherin function also promoted transmigration in response to fMLP (not shown). Under the conditions of VE-cadherin inhibition, there was no increased binding of cells to the filters (not shown). This suggests that it is not matrix exposure which contributes to the enhanced transmigration, but rather the lack of endothelial barrier function resulting from inhibition of VE-cadherin-mediated adhesion. In contrast to VE-cadherin, inhibition of endothelial PECAM-1 using a blocking monoclonal antibodies (HEC65 and HEC170) inhibited neutrophil transmigration in response

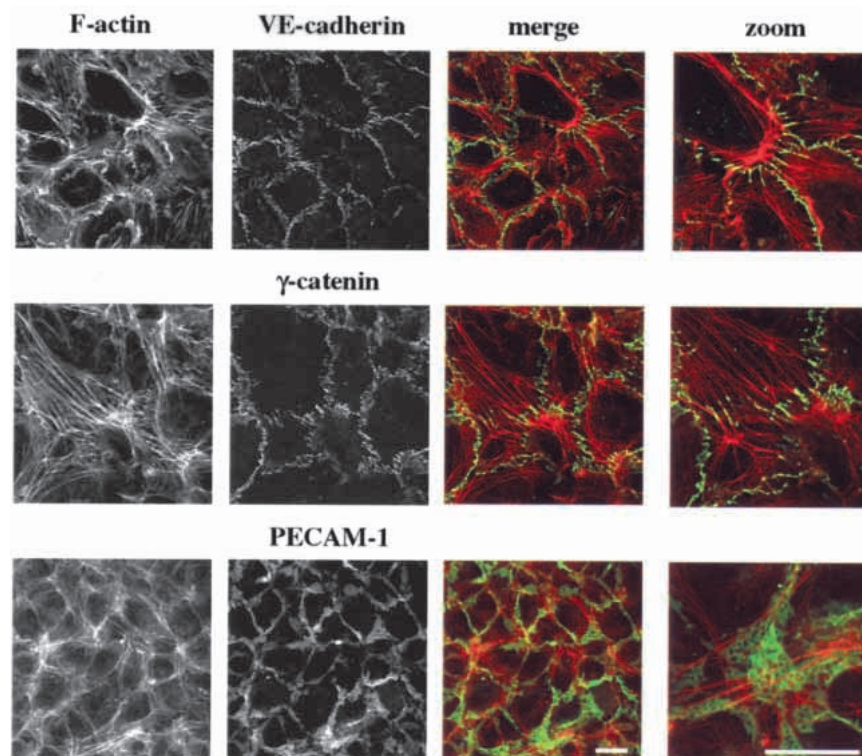


Fig. 1. Localization of VE-cadherin, γ -catenin and PECAM-1 in HUVEC. Endothelial cells were grown to confluency on fibronectin-coated glass coverslips, and the different proteins and F-actin were visualized by double immunofluorescence as described in Materials and Methods. The overlays show F-actin in red and the different proteins in green: colocalization appears in yellow. For each staining, a 3-fold zoom image is shown to underscore the colocalization of VE-cadherin and γ -catenin, but not PECAM-1, with the endpoints of actin stress fibers. In the PECAM-1 immunostaining, PECAM-1-negative areas are visible. Bars, 25 µm.

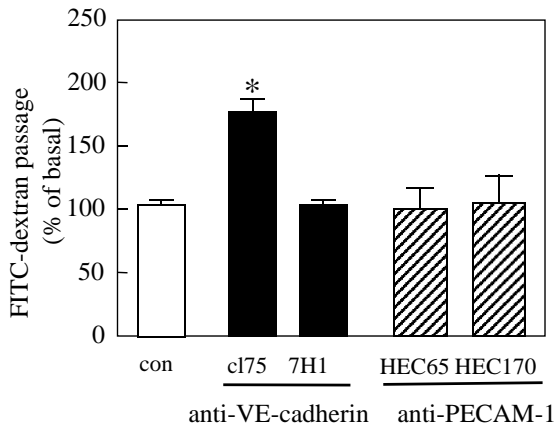


Fig. 2. Permeability of HUVEC monolayers to FITC-dextran. Cells were grown to confluency on fibronectin-coated Transwell filters, were pretreated with the antibodies (10 µg/ml) and were incubated for 2 hours with the FITC-dextran 3000. Next, the fluorescence in the lower compartment was measured in a fluorimeter (λ_{EX} 485 nm; λ_{EM} 525 nm) and expressed as percentage of basal fluorescence. The c175 anti-VE-cadherin, but not its isotype control 7H1, increased monolayer permeability. PECAM-1 antibodies had no effect. * $P < 0.05$.

to fMLP (Fig. 3B), in agreement with published data (Muller et al., 1993; Vaporciyan et al., 1993).

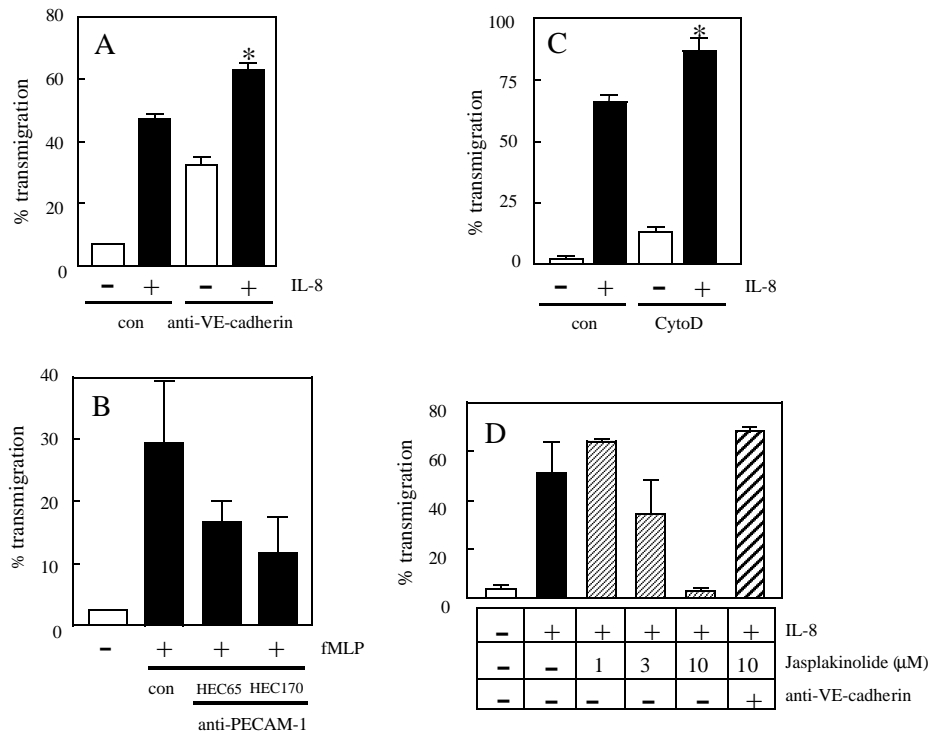
To further establish the role for the connection between VE-cadherin and the actin cytoskeleton in the control of neutrophil transendothelial migration, we pretreated endothelial monolayers with the actin disrupting agent cytochalasin D and the cell permeant, actin-assembly-promoting peptide Jasplakinolide (Bubb et al., 1994). Pretreatment of the monolayers with cytochalasin D promoted basal and IL-8-

induced neutrophil transmigration (Fig. 3C). This was most likely due to a loss of monolayer integrity, since parallel morphological analysis showed that cytochalasin D induced a disorganization of the actin cytoskeleton accompanied by the formation of F-actin aggregates, and caused loss of junctional VE-cadherin distribution and reduced cell cell contact. Moreover, no increased binding to the filters was observed (data not shown). In contrast to cytochalasin D, a brief pretreatment with Jasplakinolide dose-dependently inhibited IL-8-induced neutrophil transmigration (Fig. 3D). Jasplakinolide did not interfere with the adhesion of the neutrophils to the endothelial monolayers (not shown). Immunocytochemical analysis showed that Jasplakinolide induced in particular an increase in the F-actin content at the cell cortices, enhanced VE-cadherin staining at cellular junctions but did not induce changes in the diffuse distribution of ICAM or E-selectin (not shown). The inhibitory effect of Jasplakinolide on neutrophil transmigration was alleviated when the monolayers were pretreated with the inhibitory VE-cadherin antibody (Fig. 3D). Thus, the block in neutrophil transmigration, resulting from enhanced actin polymerization in the endothelial cells, is dependent on VE-cadherin and suggests that increased F-actin content promotes VE-cadherin-mediated cell-cell adhesion.

Inhibition of VE-cadherin causes redistribution of the protein and cytoskeletal remodeling

To explain the increased monolayer permeability induced by the c175 antibody, we analysed the distribution of F-actin and VE-cadherin in the endothelial cells. The c175 antibody, but not the 7H1 isotype control, induced a gross reorganization of the actin cytoskeleton. This was reflected by a reduction in the actin stress fiber content and a relative increase in the amount of cortical actin (Fig. 4A). Immunostaining showed VE-cadherin to be no longer localized to sites of cell-cell contact,

Fig. 3. Role of VE-cadherin in the transendothelial migration of neutrophils. HUVEC were grown to confluency on 3 µm-pore Transwell filters and were pretreated with antibodies or actin-modifying reagents. Neutrophil transmigration was measured as described in Materials and Methods. (A) The inhibitory antibody to VE-cadherin (c175, 10 µg/ml) enhanced basal and IL-8 (10^{-8} M)-induced transmigration. (B) In contrast, antibodies to CD31/PECAM (HEC 65 and HEC170, 10 µg/ml) inhibit transmigration of neutrophils. (C) Disruption of the endothelial actin cytoskeleton by cytochalasin D (CytoD; 2 µM, 30 minutes) promotes IL-8-induced transmigration. (D) Increased actin polymerization, induced by Jasplakinolide (10 µM, 10 minutes) inhibits IL-8 induced neutrophil transmigration. This inhibitory effect was alleviated when VE-cadherin was blocked by the c175 antibody (1 hour pretreatment, 10 µg/ml). * $P < 0.05$.



but to be diffusely distributed. In addition, cell-cell adhesion was significantly reduced, retraction fibers could be observed and gaps in the monolayer were apparent (Fig. 4A). Similarly, incubation with a different anti-VE-cadherin antibody (TEA1-31) reduced proper cell-cell adhesion and induced loss of junctional localization of VE-cadherin. Complementary FACS analysis showed that, following treatment of endothelial cells with the c175 antibody, the VE-cadherin protein remained present at the cell surface (Fig. 4B), indicating that it is its diffuse distribution, rather than internalization, which causes the loss of cell-cell adhesion. In contrast to VE-cadherin, antibodies to PECAM-1 did not affect the morphology of endothelial monolayers.

These data show that, although the endothelial cell-cell junctions contain many different proteins that may all be involved in the maintenance of strong cell-cell adhesion and the control of monolayer permeability, inhibition of VE-cadherin is already sufficient to disrupt monolayer integrity. In addition, these experiments underscore the functional difference between VE-cadherin and PECAM-1 in the control of permeability and of transendothelial migration. It is plausible to assume that this may be the result of the differential association of VE-cadherin and PECAM-1 with the actin cytoskeleton.

Cytoskeletal rearrangements modify VE-cadherin localization and function

The above results show that inhibition of VE-cadherin function affects the proper organization of the endothelial actin cytoskeleton and leads to reduced cell-cell adhesion. Conversely, changes in the actin cytoskeleton, e.g. induced by extracellular agonists, may modify VE-cadherin function thereby altering endothelial cell-cell adhesion and monolayer permeability, which is known to be controlled by actomyosin-based contractility (Van Hinsbergh, 1997). Regulation of this contractility can be mediated by changes in cAMP levels (Ding et al., 1994) or by the small GTPase p21Rho (Ridley and Hall, 1992; Hall, 1998). Stimulation of the β_2 -adrenergic receptor in endothelial cells with isoproterenol to increase cAMP levels, induced a marked cell flattening and a clear reduction in the amount of actin stress fibers accompanied by an increase in cortical actin. Together with the loss of stress fibers, VE-cadherin distribution at the cellular junctions was altered from a striped into a linear distribution, indicating that the normal distribution of VE-cadherin at the intercellular junctions is dependent on the presence of stress fibers. PECAM-1 distribution at the broad areas of cell-cell contact remained unaltered (Fig. 5A).

The reduction in monolayer permeability for FITC-dextran which is induced by β_2 -adrenergic receptor activation (Fig. 5B) could be restored by the subsequent addition of the VE-cadherin antibody. Moreover, when permeability was initially induced by inhibition of VE-cadherin, activation of the β_2 -adrenergic receptor had no longer any reducing effect (Fig. 5B). These results indicate that the reduction in endothelial permeability, induced by agonists that increase intracellular cAMP levels, may be due to enhanced VE-cadherin-mediated cell-cell adhesion. The present data do not exclude that this is an indirect effect, induced by the isoproterenol-induced remodeling of the actin cytoskeleton.

The small GTPase p21Rho is required for actin stress fiber

formation in response to extracellular stimuli and has also been implicated in the organization of cadherin-based cell-cell adhesion in epithelial cells (Braga et al., 1997; Takaishi et al., 1997). Inactivation of Rho by pretreatment of the endothelial cells with the C3 exoenzyme caused cytoskeletal reorganization, mainly resulting in the loss of cytoplasmic F-actin staining, leading to partial loss of cell-cell adhesion (Fig. 6). VE-cadherin distribution in these C3-treated cells was diffuse and the protein appeared no longer associated with cell-cell junctions, not even at sites where cells were still in contact. PECAM-1, however, was still detectable at sites of (partial) cell-cell contact (Fig. 6). Additional FACS analysis showed that C3 treatment did not significantly reduce VE-cadherin or PECAM-1 expression on the endothelial cells (control VE-cadherin mean fluorescence intensity (MFI)= 293, VE-cadherin + C3: MFI= 274; PECAM: control MFI=1374, PECAM + C3: MFI=1223, data are mean of duplicates). Together, these data implicate the small GTPase Rho in the formation of endothelial cell-cell junctions and in the proper distribution of VE-cadherin, and underscore the close functional and spatial connection between VE-cadherin and the actin cytoskeleton.

DISCUSSION

The intercellular junctions of endothelial cells have an important barrier function regulating the permeability to small molecules and even to cells, as in the event of leukocyte transendothelial migration. A variety of proteins that may all play a role in the maintenance of barrier function is concentrated at these cell-cell junctions. These proteins include occludins, desmoplakins, connexins, integrins, cadherins and PECAM-1 (Dejana et al., 1996; Lampugnani and Dejana, 1997). The present results corroborate the notion that VE-cadherin is one of the main components of the endothelial cell-cell junction which determines the strength of cell-cell adhesion and dictates monolayer properties.

Immunocytochemical analysis showed a colocalization of the cadherin-catenin-complex with the endpoints of actin stress fibers, suggestive for a functional connection between VE-cadherin and the actin cytoskeleton. In line with this, inhibition of VE-cadherin function induced a reorganization of the actin cytoskeleton leading to a loss of junctional VE-cadherin localization, reduced cell-cell adhesion, increased permeability, and enhanced neutrophil transmigration.

Conversely, modulation of the endothelial actin cytoskeleton was found to alter VE-cadherin function and localization. Increased actin assembly blocked neutrophil transmigration in a VE-cadherin-dependent fashion. In addition, β_2 -adrenergic receptor stimulation altered cytoskeletal reorganization and VE-cadherin distribution and reduced monolayer permeability which could be reversed by inhibition of VE-cadherin. Finally, inactivation of p21Rho caused cell flattening, loss of cytoplasmic F-actin staining, a concomitant loss of proper VE-cadherin localization and reduced cell-cell adhesion. The latter result likely explains the endothelial hyperpermeability that was induced upon inactivation of p21Rho by glucosylation (Hippenstiel et al., 1997).

The remodeling of the endothelial actin cytoskeleton and the loss of cell-cell contact which resulted from the inhibition of

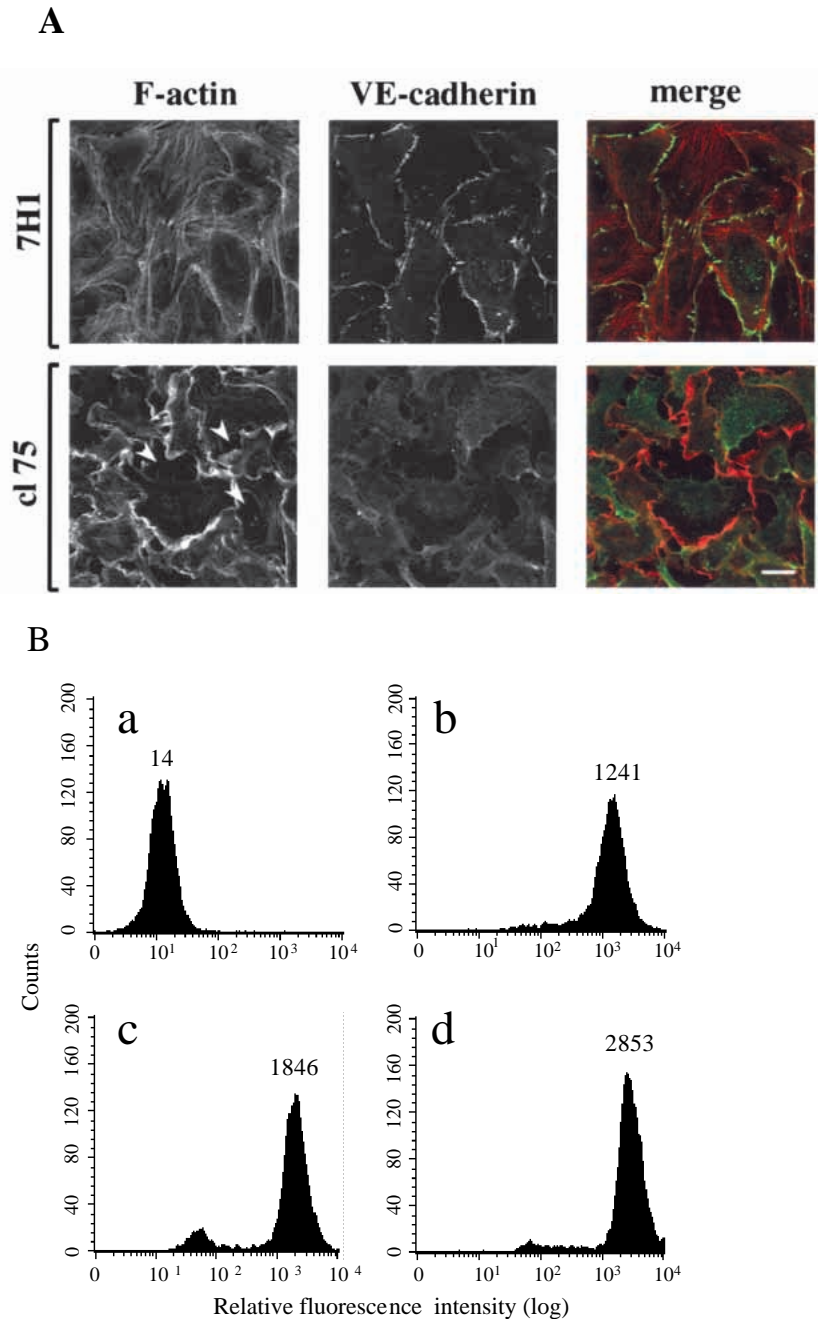


Fig. 4. Inhibition of VE-cadherin alters VE-cadherin distribution and induces cytoskeletal reorganization. (A) HUVEC were grown to confluency on glass coverslips and were treated for 1 hour with the anti-VE-cadherin antibodies 7H1 and c175. Next the cells were fixed and processed for immunofluorescence to detect VE-cadherin and F-actin. Whereas the 7H1 antibody did not affect the morphology of the cells or the distribution of VE-cadherin, the c175 antibody caused reorganization of the actin cytoskeleton as revealed by the loss of stress fibers and a relative increase in cortical actin, and induced the formation of gaps in the monolayer (arrowheads). Moreover, junctional localization of VE-cadherin was lost. Bar, 25 μ m. (B) HUVEC were incubated for 1 hour at 37°C with medium (a), the 7H1 (c) or the c175 antibody (b,d) and then processed for FACS staining using PE-labelled anti-IgG antibody (a,b) or first with the c175 (c) or the 7H1 (d) antibodies, followed by the PE-labelled anti-IgG antibody. Mean fluorescent intensities (MFI) are shown in the graphs. The results show that VE-cadherin remains present at the cell surface following incubation with the c175 antibody. The higher MFI values in c and d compared to b are due to simultaneous detection of the two antibodies with non-overlapping epitopes.

VE-cadherin likely explains the concomitant increase in monolayer permeability and neutrophil transendothelial migration. These results are in line with those from Gotsch et al. (1997) and Gulino et al. (1998) who used a monoclonal, respectively, polyclonal anti-VE-cadherin antibody to block cadherin function. Neither the anti-VE-cadherin isotype control nor the PECAM-1 antibodies altered permeability, showing that the effect was specific for the VE-cadherin protein, and for the epitope recognized by the c175 and TEA1-31 antibodies.

The role for actomyosin-based contractility in the control of monolayer permeability by extracellular agonists (e.g. thrombin, histamine; Moy et al., 1993; Rabiet et al., 1996; Vouret-Craviari et al., 1998) is well established and involves

activation of the calcium/calmodulin-dependent kinase myosin light chain kinase (MLCK; Moy et al., 1993; Goeckeler and Wysolmerski, 1995; van Hinsbergh, 1997). MLCK activation may also be important during leukocyte transmigration as several recent studies have reported activation of MLCK and induction of actin filaments in endothelial cells following adhesion of neutrophils (Hixenbaugh et al., 1997; Lorenzon et al., 1998; Saito et al., 1998). Possible effects on VE-cadherin were not addressed in these studies. In other reports, however, loss of VE-cadherin function as a result of neutrophil or tumor cell adhesion to the endothelial cells has been suggested (Del Maschio et al., 1996; Lewalle et al., 1997), but interpretation of (part of) these results has been obscured by neutrophil-derived proteolytic enzymes (Moll et al., 1998). Sandig et al.

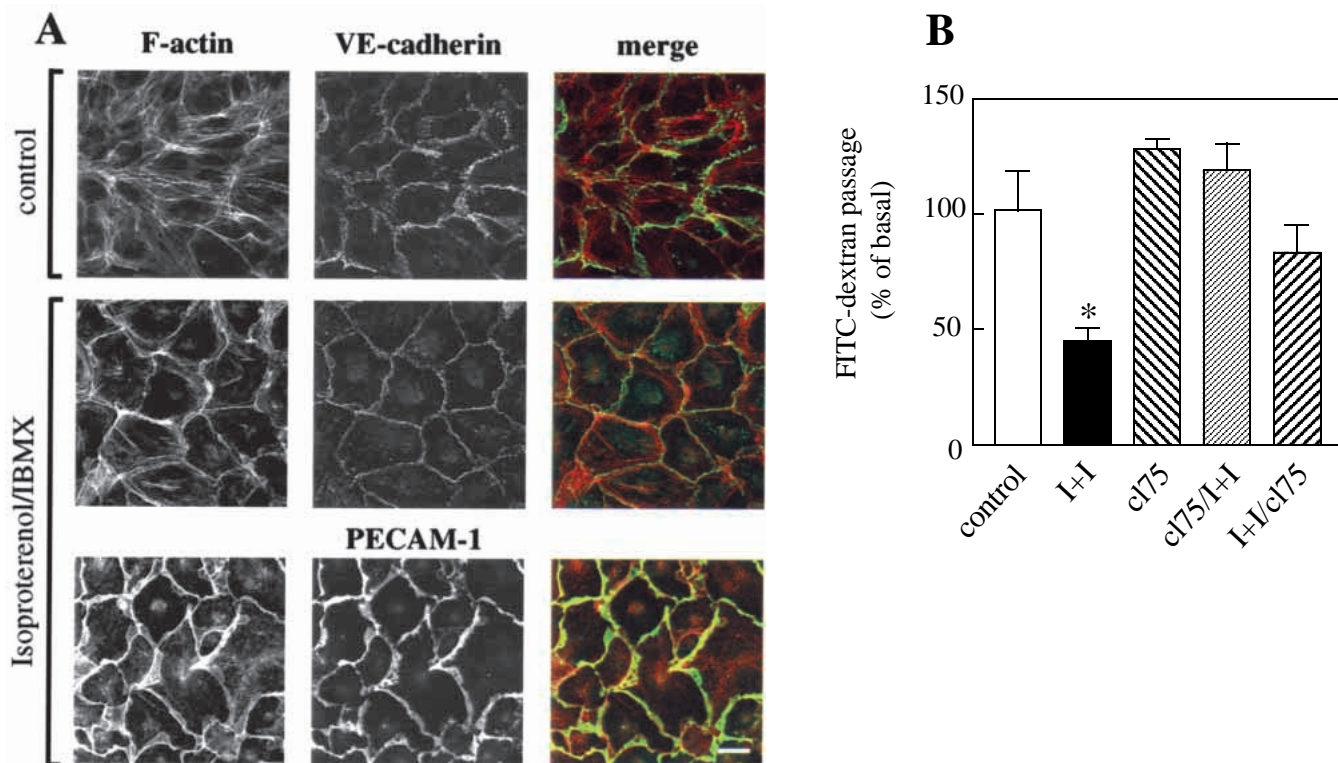


Fig. 5. β_2 -adrenergic receptor activation alters VE-cadherin distribution and monolayer permeability. (A) HUVEC were cultured on glass coverslips and incubated with the carrier DMSO (control) or stimulated with 10 μ M isoproterenol in the presence of 1 mM IBMX. Next the cells were fixed and processed for double immunofluorescence to detect VE-cadherin, PECAM-1, and F-actin. β_2 -adrenergic receptor activation induced a loss of actin stress fibers and a relative increase in cortical actin. VE-cadherin distribution became linear under these conditions, whereas PECAM-1 distribution remained unaltered and was present at the broad areas of cell-cell contact. Bar, 25 μ m. (B) Monolayer permeability to FITC-dextran 3000 was measured following pretreatment of the endothelial cells with isoproterenol/IBMX (I+I); with the VE-cadherin antibody c175 (1 hour, 10 μ g/ml); c175 followed by I+I; I+I followed by c175. The isoproterenol-mediated reduction in permeability is prevented by anti-VE-cadherin antibody treatment; moreover, the antibody largely restores isoproterenol-mediated reduced permeability. * $P < 0.05$.

(1997) described unaltered VE-cadherin localization during monocyte transendothelial migration and proposed a role for cadherin-mediated interactions between endothelial cells and transmigrating monocytes. Our data, however, show enhanced

migration in the absence of VE-cadherin function and therefore do not support a role for cadherin-based interactions with leukocyte adhesion molecules. Our results rather support the idea that cadherin function needs to be transiently reduced to

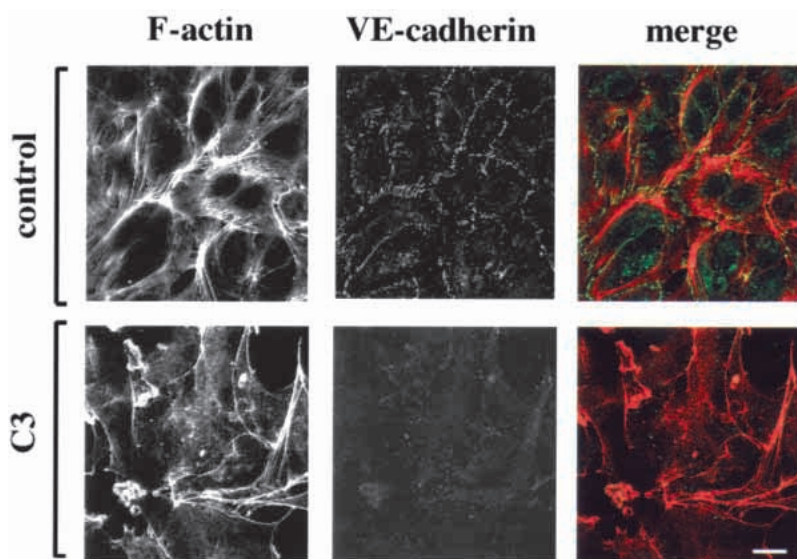


Fig. 6. Inactivation of p21Rho disrupts junctional VE-cadherin distribution. Primary HUVEC, grown on glass coverslips, were treated for 18 hours with 5 μ g/ml C3 exoenzyme to inactivate p21Rho and were immunostained for VE-cadherin, PECAM-1 and F-actin. Following C3 treatment, VE-cadherin staining was diffuse and the protein was no longer present at the sites of cell-cell contact. In addition, gaps in between the cells were apparent, indicative of reduced cell-cell adhesion. In contrast, PECAM-1 could still be detected at remaining sites of cell cell contact. Bar, 25 μ m.

allow efficient leukocyte transmigration (Del Maschio et al., 1996).

Cadherin-mediated cell-cell adhesion in endothelial cells has a similar molecular basis as in epithelial cells (Gumbiner, 1996; Lampugnani and Dejana, 1997). However, the role of the cadherins in these cell types is different. Whereas loss of VE-cadherin function is sufficient to induce loss of barrier function of endothelial monolayers, blocking E-cadherin function by prolonged culture of epithelial cells in the presence of the DECMA-1 antibody (Vestweber and Kemler, 1985) did not alter epithelial monolayer permeability (P. L. Hordijk and F. P. J. Mul, unpublished observations), whereas this antibody does inhibit E-cadherin-mediated cell-cell adhesion (Hordijk et al., 1997). This result shows that, in contrast to endothelial monolayers, epithelial monolayer permeability is not solely dependent on cadherin function.

Together, the present results suggest that actin polymerization regulates the strength of VE-cadherin-mediated cell-cell adhesion. Moreover, proper VE-cadherin localization is controlled by the organization of the actin cytoskeleton and is in particular dependent on the presence of actin stress fibers. On the other hand, loss of VE-cadherin function results in remodeling of the actin cytoskeleton. Thus, these data show the morphological and functional consequences of the bidirectional signaling between the cadherin-catenin complex and the actin cytoskeleton in endothelial cells. The results further indicate that it is possible to interfere with endothelial cell-cell adhesion by receptor activation or by means of actin-modifying agents thereby altering endothelial properties such as permeability and leukocyte transmigration. These results may find future applications in the treatment of pathology involving edema and/or excessive leukocyte infiltration.

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