Reactive oxygen species mediate Rac-induced loss of cell-cell adhesion in primary human endothelial cells

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Summary

The integrity of the endothelium is dependent on cell-cell adhesion, which is mediated by vascular-endothelial (VE)-cadherin. Proper VE-cadherin-mediated homotypic adhesion is, in turn, dependent on the connection between VE-cadherin and the cortical actin cytoskeleton. Rho-like small GTPases are key molecular switches that control cytoskeletal dynamics and cadherin function in epithelial as well as endothelial cells. We show here that a cell-penetrating, constitutively active form of Rac (Tat-RacV12) induces a rapid loss of VE-cadherin-mediated cell-cell adhesion in endothelial cells from primary human umbilical veins (pHUVEC). This effect is accompanied by the formation of actin stress fibers and is dependent on Rho activity. However, transduction of pHUVEC with Tat-RhoV14, which induces pronounced stress fiber and focal adhesion formation, did not result in a redistribution of VE-cadherin or an overall loss of cell-cell adhesion. In line with this observation, endothelial permeability was more efficiently increased by Tat-RacV12 than by Tat-RhoV14.

The loss of cell-cell adhesion, which is induced by Tat-RacV12, occurred in parallel to and was dependent upon the intracellular production of reactive oxygen species (ROS). Moreover, Tat-RacV12 induced an increase in tyrosine phosphorylation of a component the VE-cadherin–catenin complex, which was identified as α-catenin. The functional relevance of this signaling pathway was further underscored by the observation that endothelial cell migration, which requires a transient reduction of cell-cell adhesion, was blocked when signaling through ROS was inhibited.

In conclusion, Rac-mediated production of ROS represents a previously unrecognized means of regulating VE-cadherin function and may play an important role in the (patho)physiology associated with inflammation and endothelial damage as well as with endothelial cell migration and angiogenesis.

Key words: pHUVEC, Rac, Rho, Reactive oxygen species, VE-cadherin

Introduction

The endothelial lining of the vascular system represents an important barrier to plasma proteins, solutes and leukocytes. Vascular integrity is largely dependent on endothelial cell-cell adhesion mediated by vascular endothelial (VE)-cadherin (Cadherin-5, CD144) (Carmeliet, 2000; Dejana et al., 1999). VE-cadherin is a transmembrane glycoprotein that is complexed via its cytoplasmic tail to various proteins, such as the armadillo-family members β- or γ-catenin, which are in turn associated with α-catenin, which links the complex to the actin cytoskeleton (Lampugnani et al., 1995). In addition, VE-cadherin is associated with p120 catenin, which was recently shown to act as a negative regulator of the small GTPase Rho (Noren et al., 2000).

Like the other cadherins, VE-cadherin mediates calcium-dependent, homophylic intercellular adhesion and is an important regulator of endothelial permeability (Corada et al., 1999; Hordijk et al., 1999). Inhibition of VE-cadherin-mediated cell-cell adhesion has pronounced effects on the organisation of the endothelial actin cytoskeleton and vice versa (Hordijk et al., 1999; Wojciak-Stothard et al., 1998). Transfection studies have shown that VE-cadherin is involved in endothelial cell migration (Breviario et al., 1995) and survival (Carmeliet et al., 1999) and appears to be required for the organisation of vascular structures in embryoid bodies, angiogenesis and tumor growth (Liao et al., 2000). The mechanisms that control the function of VE-cadherin are not well understood, but they have been suggested to involve indirect signaling through changes in the actin cytoskeleton and more direct signaling through tyrosine phosphorylation of VE-cadherin or its associated proteins (Esser et al., 1998; Lampugnani et al., 1997).

Recent studies have underscored the important role for the actin cytoskeleton in regulating cadherin function and cell-cell adhesion in epithelial and endothelial cells. Consequently, Rho family GTPases have been identified as regulators of cadherin-based cell-cell adhesion. In epithelial cells, Rac, Rho and CDC42 have all been implicated in the formation and control of E-cadherin-mediated cell-cell adhesion (Braga et al., 1997; Braga et al., 1999; Hordijk et al., 1997; Takaishi et al., 1997). In endothelial cells, the functional link between Rho-like GTPases and VE-cadherin function is less clear. Inhibition of Rho-dependent contractility has been shown to prevent receptor-mediated increases in endothelial permeability

(Nieuw Amerongen et al., 1998), whereas others have shown that activation of Rho using bacterial toxins was not sufficient to perturb endothelial integrity (Vouret-Craviari et al., 1999). Dominant-negative Rac (RacN17) has been described as blocking as well as promoting thrombin-induced permeability in transfected HUVEC (Vouret-Craviari et al., 1998; Wojciak-Stothard et al., 2001). In both of these studies, active Rac (RacV12) was found to mimic the effects of thrombin in stimulating permeability. In contrast to this studies, Braga et al. (Braga et al., 1999) have described endothelial cell-cell adhesion as independent from either Rho or Rac activity. However, this study was performed using unstimulated cells, which may explain the different effects of the Rho and Rac GTPases.

In previous work, we showed that activation of Rac promotes E-cadherin function in normal and Ras-transformed epithelial cells (Hordijk et al., 1997). We therefore investigated whether introduction of constitutively active Rac would also affect the localisation and function of VE-cadherin in primary human endothelial cells. Since these cells are notoriously difficult to manipulate using classical transfection or retroviral transduction techniques, we developed cell-penetrating variants of Rho-like GTPases by fusion with the cell-penetrating sequence of HIV-Tat (Nagahara et al., 1998). The main advantages of using such cell-penetrating GTPases are that one can analyse cellular responses in primary human cells directly after addition of the proteins, which is comparable to adding receptor agonists, and that all cells in the culture are transduced, which allows the combination of functional and biochemical assays with morphological analysis.

Using this approach we show here that, in contrast to epithelial cells, introduction of active Rac in primary human endothelial cells disturbs VE-cadherin localisation and reduces cell-cell adhesion. This was paralleled by and dependent on the synthesis of reactive oxygen species (ROS) and was also accompanied by tyrosine phosphorylation of the VE-cadherin complex. This mode of regulation is likely to be relevant during a variety of (patho)physiological processes, such as inflammation, endothelial damage following ischemia, endothelial cell migration and angiogenesis.

Materials and Methods

Antibodies

Mouse monoclonal antibodies (Mabs) against VE-cadherin (cl75), vinculin, α- or β-catenin or phosphotyrosine (PY-20) were from Transduction Laboratories (Becton Dickinson Copany, Amsterdam, The Netherlands). VE-cadherin antibody 7H1 was from Pharmingen (San Diego, CA. USA). Texas Red phalloidin, FITC-dextran 3000 and Alexa-488 goat-anti-mouse-Ig secondary antibody were all from Molecular Probes (Leiden, The Netherlands). Goat-anti-mouse Ig conjugated to horseradish peroxidase was purchased from the CLB (Amsterdam, The Netherlands). *Clostridium botulinum* exoenzyme C3 was obtained from Kordia Laboratory supplies (Leiden, The Netherlands).

Cell culture

Human endothelial cells were harvested from umbilical veins (HUVECs) as described previously (Brinkman et al., 1994) and maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated human serum (HSA, CLB), 2 mM glutamine (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco) in fibronectin-coated cultures flasks. The cells were used from passages two to four.

NIH3T3 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) containing 10% heat-inactivated foetal calf serum (FCS, Gibco), 2 mM glutamine and 100 units/ml penicillin and 100 μg/ml streptomycin. Ras-transformed epithelial cells, Madin Darby Canine Kidney-f3 (MDCK-f3), were cultured in Dulbecco's Modified Epithelial Medium (DMEM, Gibco) containing 10% heat-inactivated fetal calf serum (FCS, Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin.

Retroviral tranduction and transfection assays

Immortalized HUVEC were transduced as described (Michiels et al., 2000) with a MMLV-based amphotrophic retrovirus that contained the GFP-human β-actin fusion protein. The retroviral construct was generated by cloning the GFP actin from the pEGFP-actin vector (Clontech, Palo Alto, USA) using *Nhe*I-*Bam*HI restiction digestion into pBluescript and subsequently *Not*I (blunted)-*Eco*RI into the *Swa*I-*Eco*RI sites of the retroviral vector.

Protein purification and transduction

To produce Tat-fusion proteins, PCR products encoding human Rac1 (V12 and N17 mutants) and RhoA (V14 and N19 mutants) were cloned as *Kpn*I/*Eco*RI fragments into the pTat-HA factor (Nagahara et al., 1998), sequenced and transformed into the BL21(DE3) strain. Rac primers: forward, GATCGGTACCCAGGCCATCAAGTGTG-TGGT; reverse, GATCGAATTCTTACAAACAGCAGGCATTTTC-TC; Rho primers: forward, 5′-GATCGGTACCGCTGCCATCCGG-AAGAAACT-3′; reverse, 5′-GATCGAATTCTCACAAGACAAGG-CAACCAG-3′. Transformed bacteria were obtained from an overnight culture, resuspended and sonicated in Z-buffer (8 M Urea, 100 mM NaCl and 20 mM Hepes, pH 8.0). Cleared lysates, produced by the addition of 20 mM imidazole, were loaded onto a Ni-NTA column (Qiagen) as described (Nagahara et al., 1998). Tat-fusion proteins were eluted with 1 M imidazole in Z buffer, diluted five times with 20 mM Hepes buffer pH 8.0 and applied to a Source 30Q column (Pharmacia Biotech, Uppsala, Sweden). After washing, bound proteins were eluted with 1 M NaCl, desalted on PD-10 columns with PBS with 1 mM Ca^{2+}, snap frozen in 10% (v/v) glycerol and stored at –80°C.

For transduction, proteins were added directly to the cells in normal culture medium at a final concentration of 50 nM. For controls, we used the Tat-PTD peptide (YGRKKRRQRRR), which was dissolved in PBS/1 mM Ca^{2+}.

Permeability assays

Permeability of pHUVEC monolayers, cultured for 4-5 days on Transwell filters (0.4 μm pore size, 12 mm diameter; Costar, Cambridge, MA, USA), was assessed with FITC-labeled dextran (Hordijk et al., 1999). Phalloidin staining of cells on a filter, cultured in parallel, was used to confirm confluency of the monolayers used in the permeability assays. The endothelial cell monolayers were preincubated for 30 minutes with 50 nM of Tat-RacV12, Tat-RhoV14 or an antibody to VE-cadherin as a positive control (10 μg/ml; cl75). FITC-dextran 3000 (10 μg/ml; Molecular Probes, Leiden, The Netherlands) was then added to the upper compartment, and fluorescence in the lower compartment was measured after 2 hours with a spectrofluorimeter (λ_{ex} 485 nm; λ_{em} 525 nm). Permeability of untreated monolayers was set at 100% (absolute permeability was in the range of 5-10% and depended on donor variability).

Immunocytochemistry

Cells that were cultured to subconfluency were incubated with TAT-proteins or the TAT-peptide in PBS with 1 mM Ca^{2+} for various time

periods, fixed and permeabilized with 2% paraformaldehyde and 0.5% (v/v) Triton-X100 in wash buffer (PBS containing 0.5% (v/v) HSA, 1 mM Ca^{2+}) for 20 mintes at room temperature (RT). Cells were stained with the indicated mouse monoclonal antibodies, washed and incubated with Alexa-488-conjugated goat-anti-mouse Ig antibodies in combination with Texas Red phalloidin (1 U/ml) to visualize F-actin. Images were recorded with a ZEISS LSM 510 confocal laser scanning microscope. Immunofluorescent staining for the HA-epitope confirmed protein transduction of all cells in the culture (not shown). For time-lapse confocal microscopy, cells were mounted in culture medium in a temperature-controlled incubation chamber kept at 37°C.

Endothelial cell migration assay

To monitor endothelial cell migration, pHUVECs were serum-starved overnight, detached with 5 mM EDTA and plated on fibronectin-coated Transwell filters (8 μm pore size, 6.5 mm diameter; Costar) in serum-free RPMI 1640 medium. Medium containing 0-10% FCS was added to the lower chamber and the cells were allowed to migrate for 3-5 hours. Next, the cells were fixed in 2% paraformaldehyde containing 0.5% (v/v) human serum albumin (HSA) and 1 mM Ca^{2+}. Cells from the upper compartment were removed with a cotton swab. Nuclei of migrated cells were stained with Hoechst 33258 and counted by fluorescence microscopy.

Measurement of reactive oxygen species

To measure generation of reactive oxygen species (ROS) in endothelial cells, pHUVECs cultured on fibronectin-coated glass coverslips were loaded with dihydrorodamine 123 (DHR, 30 μM; Molecular Probes) for 30 minutes, washed and subsequently transduced with the Tat-peptide or Tat-fusion proteins. Fluorescence of DHR was quantified by time-lapse confocal microscopy. Intensity values are shown as the percentage increase relative to the values at the start of the experiment.

Immunoprecipitaton and western blotting

Cells were grown to confluency in 6-well plates, washed and lysed for 10 minutes in 0.5 ml lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5 mM orthovanadate with the addition of protease inhibitor cocktail tablets (Boehringer Mannheim, Mannheim, Germany) and, where indicated, in the presence of 0.1% SDS. The lysates were precleared with 25 μl Protein A-sepharose beads (Pharmacia Biotech). Next, the lysates were incubated with 25 μl of Protein A beads, coated with 10 μg/ml VE-cadherin (7H1) or anti-α-catenin antibodies for 1 hour at 4°C under continuous mixing. The beads were extensively washed in lysis buffer, and proteins were eluted by boiling in sample buffer. Samples were run on 10% SDS-PAGE under reducing conditions, transferred onto 0.2 μm nitrocellulose filters (Schleicher & Schuell, Dassel, Germany), which were blocked with 5% dried milk protein in TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.5% (v/v) Tween-20) and incubated with the appropriate antibodies (to VE-cadherin (7H1), α-catenin or PY-20, all diluted 1:1000 in blocking buffer). This was followed by incubation with rabbit anti-mouse (RαM) IgG-HRP (1:1000, DAKO) at room temperature. The immunoreactive bands were visualised with the ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, England).

Results

Transduction of Tat-RacV12 into fibroblasts and epithelial cells

A cell-penetrating, constitutively active mutant of Rac (Tat-RacV12) was constructed by fusion of the Rac coding sequence to that of the protein transduction domain of HIV-TAT (Nagahara et al., 1998). The fusion protein was isolated as previously described for Tat-Rho proteins (Alblas et al., 2001; Chellaiah et al., 2000) and validated by transduction into NIH3T3 fibroblasts and Ras-transformed epithelial MDCKf3 cells. Time-lapse studies of NIH3T3 fibroblasts showed that Tat-Rac-V12 induced a limited and transient contractile response within 1-3 minutes, followed by the rapid formation of lamellipodia and membrane ruffles. Visualisation of the F-actin cytoskeleton and of vinculin, a marker for focal adhesions and focal contacts, showed that Tat-RacV12 induced a rapid increase in the stress fiber content of the cells, which was accompanied by formation of vinculin-containing focal adhesions (Fig. 1a,b). In addition, lamellipodia were formed along the cells' periphery, where rims of cortical actin, protruding over vinculin-containing focal contacts, were observed.

The effects of Tat-RacV12 were also tested in Ras-transformed MDCKf3 cells, which are known to revert from a fibroblastoid to a more epithelial phenotype upon expression of constitutively active Rac (Hordijk et al., 1997). Time-lapse

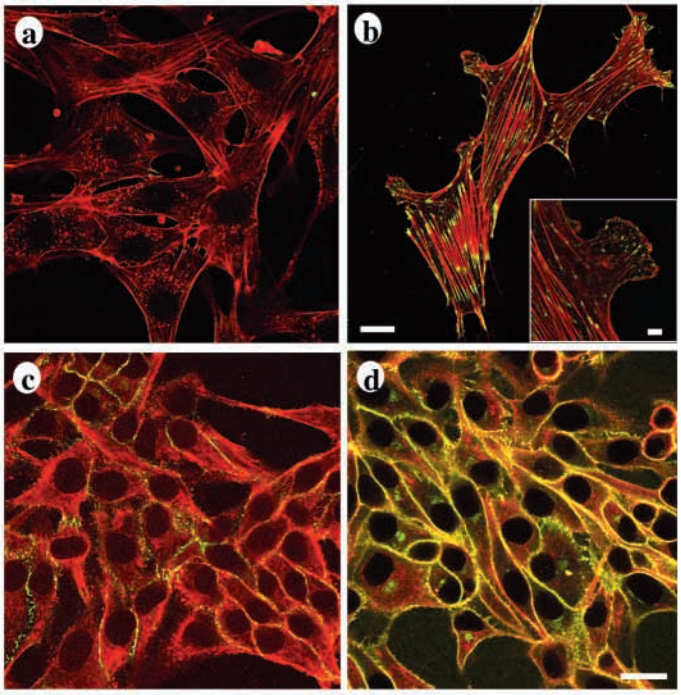

Fig. 1. Transduction of Tat-RacV12 in fibroblasts and epithelial cells. Tat-PTD (control, a) or Tat-RacV12 (b) was transduced into NIH3T3 fibroblasts for 30 minutes. Next the cells were fixed and permeabilized, (immuno)stained for actin (red) and vinculin (green) and analysed by confocal microscopy. Tat-RacV12 induced rapid formation of actin stress fibers and induced pronounced vinculin-containing focal adhesions and lamellipodia along the cells' periphery (inset). Bar, 10 μm (inset: bar, 5 μm). Ras-transformed epithelial MDCK cells were transduced with the Tat-PTD (c) or Tat-RacV12 (d), and the distribution of F-actin and β-catenin was analysed by immunocytochemistry after 3 hours. Tat-RacV12 did not induce stress fibers but recruited F-actin to the cellular cortex. In addition, β-catenin was found to be more pronounced at the sites of cell-cell contact, which is indicative of increased cell-cell adhesion. Bar, 10 μm.

analysis showed that these MDCK cells, in contrast to NIH3T3 cells, did not show either contraction or lamellipodia formation following transduction with Tat-RacV12. However, incubation with Tat-RacV12 induced a clear increase in cortical F-actin and recruitment of β-catenin to cell-cell contact sites (Fig. 1c,d). These effects were already seen after 3 hours, but were most obvious following overnight incubation, indicating that the cell-penetrating Rac protein, similar to its retrovirally expressed equivalent, promotes cell-cell adhesion in these Ras-transformed epithelial cells. These data demonstrate that protein transduction of RacV12 induces similar phenotypic changes to RacV12 proteins expressed through microinjection

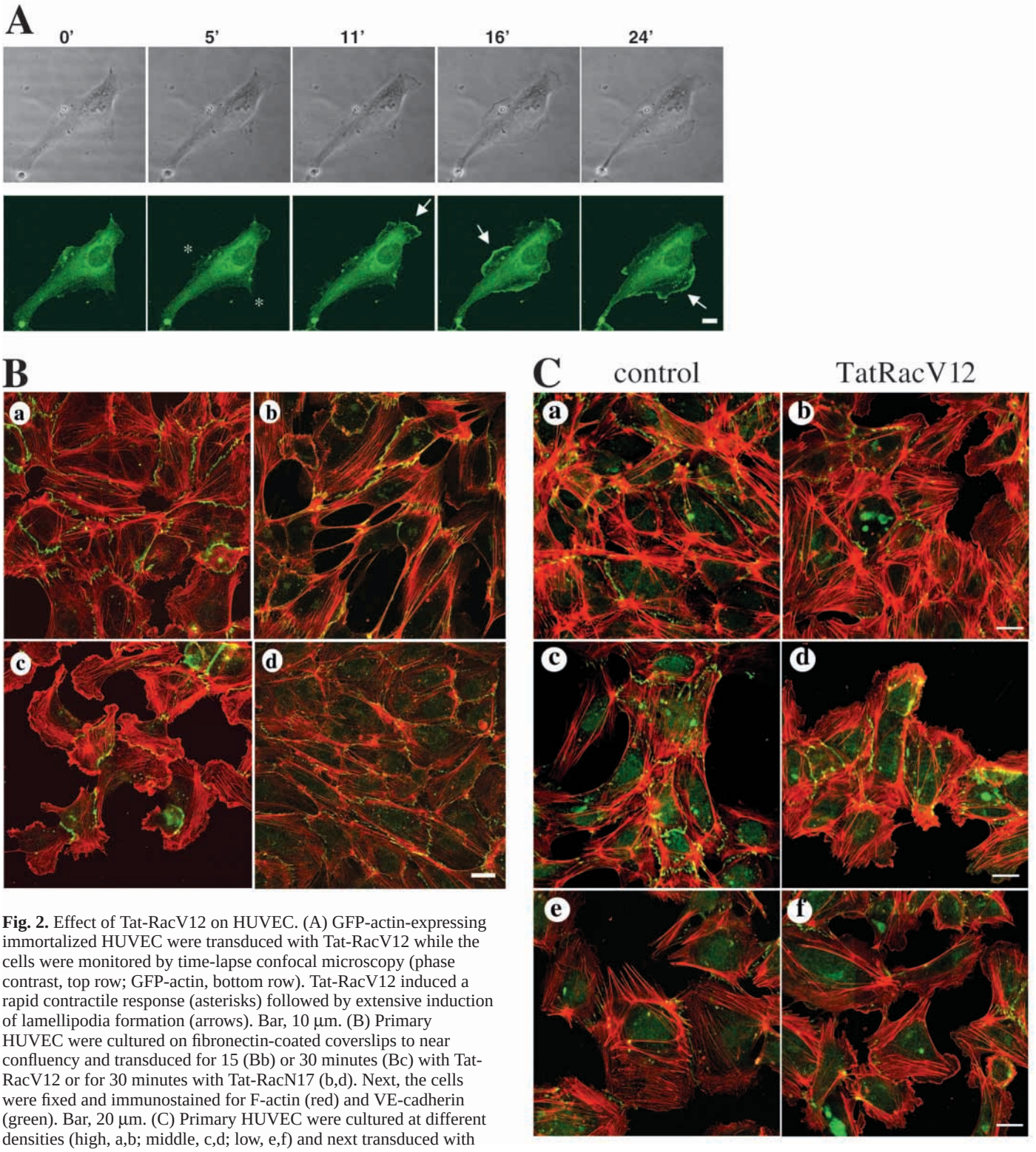

Fig. 2. Effect of Tat-RacV12 on HUVEC. (A) GFP-actin-expressing immortalized HUVEC were transduced with Tat-RacV12 while the cells were monitored by time-lapse confocal microscopy (phase contrast, top row; GFP-actin, bottom row). Tat-RacV12 induced a rapid contractile response (asterisks) followed by extensive induction of lamellipodia formation (arrows). Bar, 10 μm. (B) Primary HUVEC were cultured on fibronectin-coated coverslips to near confluency and transduced for 15 (Bb) or 30 minutes (Bc) with Tat-RacV12 or for 30 minutes with Tat-RacN17 (b,d). Next, the cells were fixed and immunostained for F-actin (red) and VE-cadherin (green). Bar, 20 μm. (C) Primary HUVEC were cultured at different densities (high, a,b; middle, c,d; low, e,f) and next transduced with Tat-RacV12 for 30 minutes. Next, the cells were fixed and immunostained for F-actin (red) and VE-cadherin (green). Bars, 20 μm.

(Ridley et al., 1992) or retroviral transduction (Hordijk et al., 1997).

Transduction of Rac proteins in primary HUVECs

Given the stimulatory effect of Rac on E-cadherin function in epithelial cells (Hordijk et al., 1997), we analysed its effect on cadherin-based adhesion and barrier function of primary HUVECs. The Tat-RacV12 protein induced pronounced but transient cellular contraction, followed by extensive cell spreading and lamellipodia formation. This was clearly visualized in immortalized HUVEC (Fontijn et al., 1995) that expressed a GFP-actin fusion protein (Fig. 2A). Immunocytochemical analysis of subconfluent pHUVEC showed that Tat-RacV12 induced endothelial contractility and intercellular gap formation after only 15 minutes of transduction (Fig. 2Bb), and prominent loss of cell-cell adhesion and formation of lammelipodia was observed after 30 minutes. Furthermore, this Tat-RacV12-induced contractility and loss of cell-cell adhesion was accompanied by a rapid increase in the levels of F-actin stress fibers and in altered VE-cadherin distribution (Fig. 2Bc). Complementary FACS analysis showed that under these circumstances the surface expression of VE-cadherin remained unaltered (not shown). In contrast, transduction of Tat-RacN17 protein did not induce significant changes in the cytoskeleton or in VE-cadherin distribution (Fig. 2Bd). When Tat-RacV12-treated cells were left overnight, VE-cadherin distribution and cell-cell adhesion were restored (not shown), indicating that the protein was not toxic to the cells and that its effects were reversible. Together, these results show that the Tat-RacV12 protein induces a rapid loss of VE-cadherin-mediated cell-cell adhesion followed by the induction of a 'Rac-phenotype', as deduced from the induction of lamellipodia.

It has been reported that control of endothelial cell-cell adhesion by Rho-like GTPases is dependent on the confluency of the cells (Braga et al., 1999). The effects of Tat-RacV12 were therefore analysed in cultures of different cell density. The data in Fig. 2C show that in confluent monolayers, Tat-RacV12 induces mainly stress fibers and small gaps in between the cells. Occasionally, formation of ruffles can also be observed. At subconfluent monolayers, the Tat-RacV12-

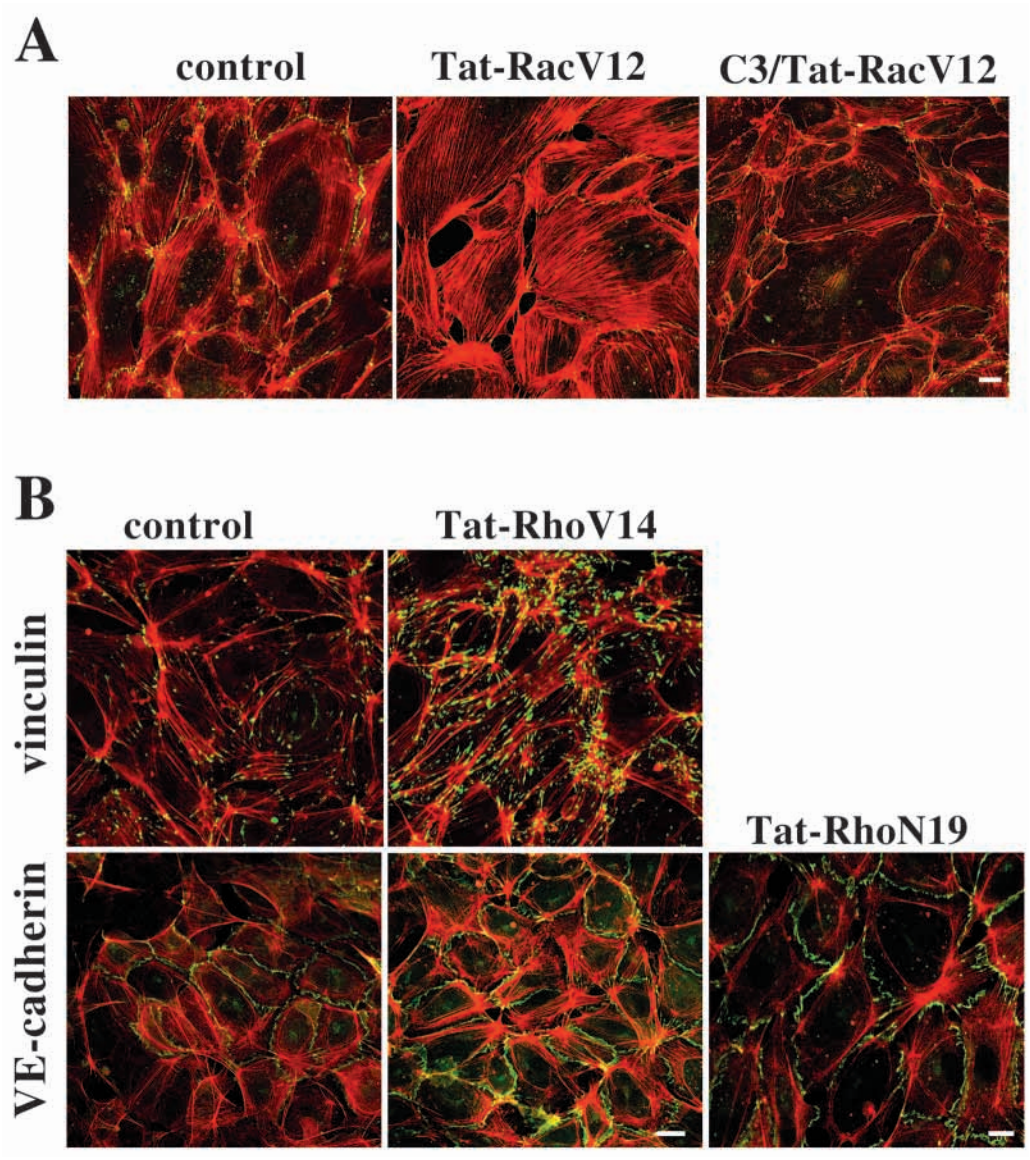

Fig. 3. Role of Rho in the control of endothelial cell-cell adhesion. (A) Primary HUVECs on fibronectin-coated coverslips were pretreated or not with the Rho-inactivating C3 exoenzyme (10 μg/ml) and subsequently transduced with the Tat PTD or Tat-RacV12 (50 nM) for 30 minutes. The cells were fixed and stained for F-actin (red) and VE-cadherin (green). Bar, 20 μm.
(B) Primary HUVEC were transduced with Tat-RhoV14 or Tat-RhoN19 for 30 minutes, fixed and stained for F-actin (red) and vinculin or VE-cadherin (green). Bars, 20 μm.

induced cellular contraction results in a more pronounced loss of cell-cell adhesion, accompanied by formation of lamellae and ruffles. Thus, the effects of Tat-RacV12, that is, induction of contractility and gap formation, seem not to be qualitatively different, but a more general loss of cell-cell adhesion and appearance of lamellae is most clear at lower densities.

Role of Rho GTPase in Rac-mediated responses

As the effects of Tat-RacV12 were accompanied by transient cellular contraction, we tested Rho-mediated contractility for a role in this response. Pretreament of pHUVECs with the C3 exo-enzyme from *Clostridium botulinum* prevented Tat-RacV12-induced stress fiber formation and loss of cell-cell adhesion (Fig. 3A), suggesting that in HUVECs, as in fibroblasts (Ridley and Hall, 1992), the Rac phenotype requires Rho activity. To assess whether the effects of Rac could be mimicked by constitutively active Rho, we transduced pHUVECs with Tat-RhoV14. The active Rho protein rapidly induced pronounced stress fiber formation and prominent focal adhesions, as revealed by vinculin staining (Fig. 3B), but did not induce significant changes in junctional VE-cadherin staining or loss of cell-cell adhesion (Fig. 3B). Small gaps in between the cells could be observed, but this effect was not comparable with the more general loss of cell-cell adhesion induced by transduction of Tat-RacV12 (compare Fig. 3B with Fig. 2A). Tat-RhoN19, the inactive mutant of Rho, had little effect on the cellular F-actin distribution and did not significantly alter VE-cadherin distribution (Fig. 3B).

To analyse whether the Tat-RacV12-induced loss of cell-cell adhesion and gap formation would result in reduced monolayer integrity we performed permeability assays. As shown in Fig. 4A, Tat-RacV12 increased endothelial monolayer permeability by 25-30% compared with basal values. Tat-RhoV14 only partially mimicked the response of Tat-RacV12 and increased permeability by approximately 15%. An antibody against VE-cadherin was used as a positive control. As shown in Fig. 4B, Tat-RacV12 also induced gap formation and loss of cell-cell adhesion in highly confluent cells that were grown on the filters. The results obtained with Tat-RacV12 and Tat-RhoV14 are in line with the immunofluorescence analysis shown in Figs 2A and 3B and indicate that, although Rho activity is required for Rac-mediated loss of cell-cell adhesion, the Tat-RhoV14 protein by itself is not sufficient to fully mimic this effect. The combination of the constitutively active Rac and Rho proteins did not result in an additional increase in the Rac-induced increase in permeability (not shown). The Tat-RacV12-mediated increase in endothelial permeability in HUVEC is in agreement with recent data from Wojciak-Stothard et al. (Wojciak-Stothard et al., 2001) who used adenovirus to express active Rac proteins in primary HUVEC.

Induction of ROS by Tat-RacV12 in primary HUVECs

In phagocytes, the Rac2 protein is essential in the synthesis of ROS through its activation of the NADPH oxidase complex (Diebold and Bokoch, 2001). Exposure to ROS, in particular H_2O_2, has been show to reduce cell-cell adhesion in epithelial cells (Volberg et al., 1992) and to affect endothelial cell function (Lum and Roebuck, 2001). Since Rac activation has also been implicated in the production of ROS in endothelial

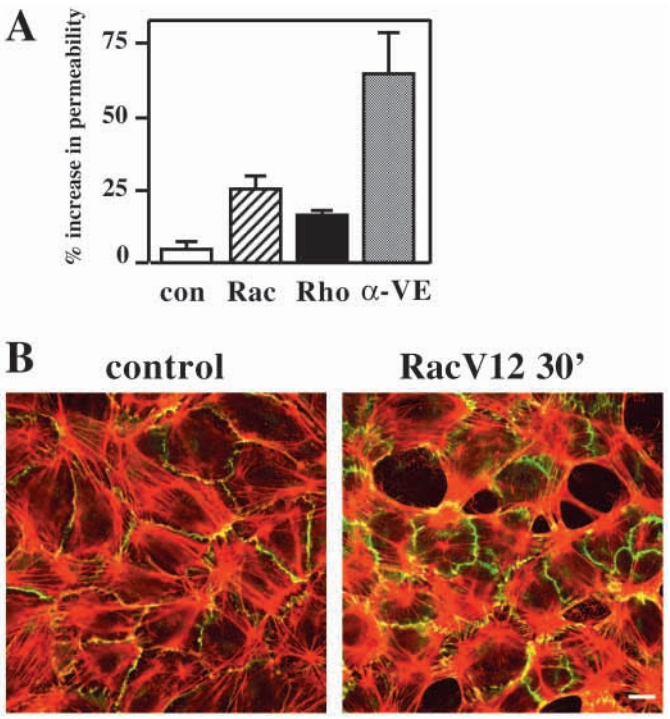

Fig. 4. Effect of Tat-RacV12 and Tat-RhoV14 on endothelial barrier function. (A) Primary HUVECs were cultured on fibronectin-coated transwell-filters until confluent, and monolayers were pretreated for 30 minutes with Tat-RacV12 and TAT-RhoV14 (both 50 nM) or an antibody against VE-cadherin that was used as a positive control. Permeability was assessed 2 hours later as described in the Materials and Methods. (B) Endothelial cells, which were cultured on fibronectin-coated transwell filters, were treated with Tat-RacV12 for 30 minutes, and cells were then stained and fixed for F-actin (red) and VE-cadherin (green). Bar, 10 μm.

cells (Deshpande et al., 2000), we analyzed whether production of ROS was induced by Tat-RacV12. As shown in Fig. 5A, Tat-RacV12 induced a rapid (within 5 minutes) increase in DHR (dihydrorodamine 123) fluorescence, indicating that ROS were formed. This effect lasted for approximately 60 minutes, after which time a decline in DHR fluorescence was observed. These kinetics paralled the effects on cell-cell adhesion. In contrast, neither the Tat-peptide nor Tat-RhoV14 (not shown) induced ROS production.

To determine whether ROS were involved in Tat-RacV12-mediated loss of cell-cell adhesion, pHUVEC were pretreated with the oxygen scavenger N-acetyl-cysteine (N-AC) and then incubated with Tat-RacV12. N-AC, which by itself left the cytoskeleton and VE-cadherin distribution unaltered, was found to prevent the Tat-RacV12-induced production of ROS (not shown) and blocked the concomitant loss of cell-cell contacts (Fig. 5Bc), indicating that ROS are essentially involved in Rac-mediated loss of cell-cell adhesion and VE-cadherin redistribution. In contrast, the induction of stress fibers was not inhibited in N-AC-pretreated cells, demonstrating that N-AC is not toxic for the cells and did not interfere with (Rho-dependent) cell signaling events (Fig. 5Bc). The addition of 1 mM H_2O_2 mimicked the Tat-RacV12-induced loss of cell-cell adhesion (not shown**)**.

Induction of tyrosine phosphorylation

The pathways that regulate VE-cadherin-mediated cell-cell adhesion from within the endothelial cells are poorly defined. Cellular contractility has been proposed to be important, but our present data indicate that the induction of Rho-mediated contractility is not sufficient to disrupt VE-cadherin function (Fig. 3B). Others have reported an important role for tyrosine phosphorylation of the VE-cadherin complex as a means to regulate cell-cell adhesion in epithelial and endothelial cells (Braga et al., 1999; Esser et al., 1998; Volberg et al., 1992). In this regard, tyrosine phosphorylation of VE-cadherin, β-catenin and γ-catenin have been reported, although the kinase that is involved in phosphorylation of the cadherin-catenin complex in endothelial cells has not been identified.

To investigate the induction of tyrosine phosphorylation, the cellular distribution of phosphotyrosine was analysed in pHUVEC that had been transduced with Tat-RacV12. The data in Fig. 6A show that Tat-RacV12 induced an increase in phosphotyrosine content, in particular at cell borders. To detect tyrosine phosphorylation at adherens junctions, phosphotyrosine was detected using a monoclonal antibody, and, owing to the lack of a suitable polyclonal VE-cadherin antibody, adherens junctions were marked using a polyclonal anti-β-catenin antibody. Colocalisation of phosphotyrosine and β-catenin could be observed following transduction with Tat-RacV12, indicating that junctional proteins become phosphorylated (Fig. 6B). To test for specific changes in tyrosine phosphorylation of the VE-cadherin complex, the fusion protein was immunoprecipitated under non-denaturing conditions following transduction of the cells with Tat-RacV12. Within 10-20 minutes, increased tyrosine phosphorylation was detected in the immunoprecipitate, albeit not of VE-cadherin itself (migrating at around 130 kDa), but of an associated protein with a molecular weight of approximately 100-110 kDa (Fig. 6C). This apparent molecular weight corresponds to that of α-catenin. Subsequent immunoprecipitation under denaturing conditions indicated tyrosine phosphorylation of α-catenin (molecular weight, 105 kDa) upon transduction of pHUVEC with Tat-RacV12 (Fig. 6D). This response was inhibited when the cells were preincubated with N-AC, indicating that the phosphorylation of α-catenin is dependent on Rac-mediated production of ROS.

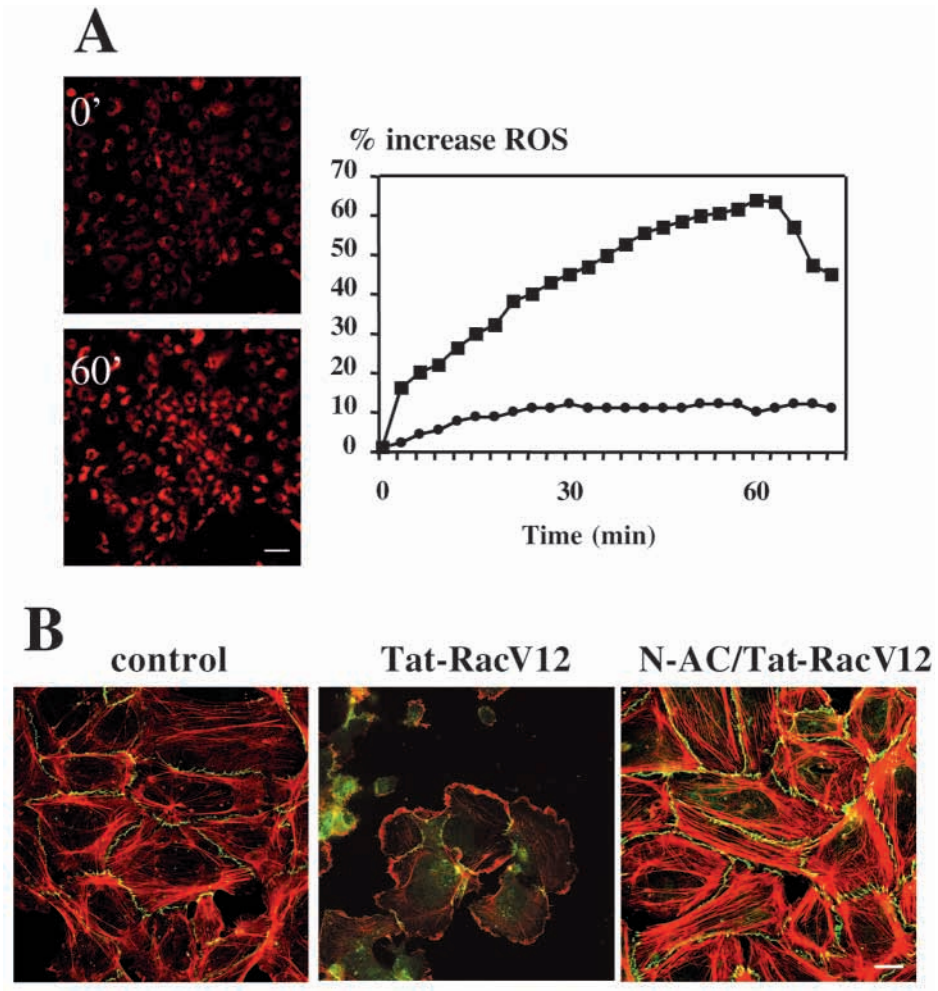

Fig. 5. Role for ROS in Tat-RacV12-mediated loss of cell-cell adhesion in pHUVEC. (A) Tat-RacV12-induced production of reactive oxygen species (ROS). Endothelial cells were loaded with the H_2O_2-sensitive dye DHR, washed, transduced with the TAT-peptide (●) or Tat-RacV12 (■, 50 nM) and monitored for DHR fluorescence using time-lapse microscopy. Images showing DHR fluorescence at t=0 minutes and t=60 minutes are shown in the panels on the left. Bar, 50 μm. The graph depicts the % increase over basal values. (B) The role of ROS in the Tat-RacV12-induced loss of cell-cell adhesion. pHUVEC were cultured in the absence (a,b) or presence (c) of 5 mM N-AC prior to transduction with Tat-RacV12 for 30 minutes (b,c). Fixed cells were stained for F-actin (red) and VE-cadherin (green). Bar, 20 μm.

To further establish the significance of Rac-mediated ROS production for endothelial cell function, we analysed migration of pHUVEC across fibronectin-coated Transwell filters. Serum increased endothelial migration five-fold after 3 hours and 15-fold after 5 hours of migration (Fig. 7). No differences were observed in the migration induced by either 5 or 10% serum. Transduction of pHUVEC with Tat-RacV12 did not promote either the spontaneous or serum-induced migration (not shown). However, pretreatment of the pHUVEC with N-AC almost completely abrogated serum-induced migration of pHUVEC, underscoring the importance of cellular ROS production for endothelial cell motility.

Discussion

The control of VE-cadherin-mediated endothelial cell-cell adhesion is important in the context of a variety of

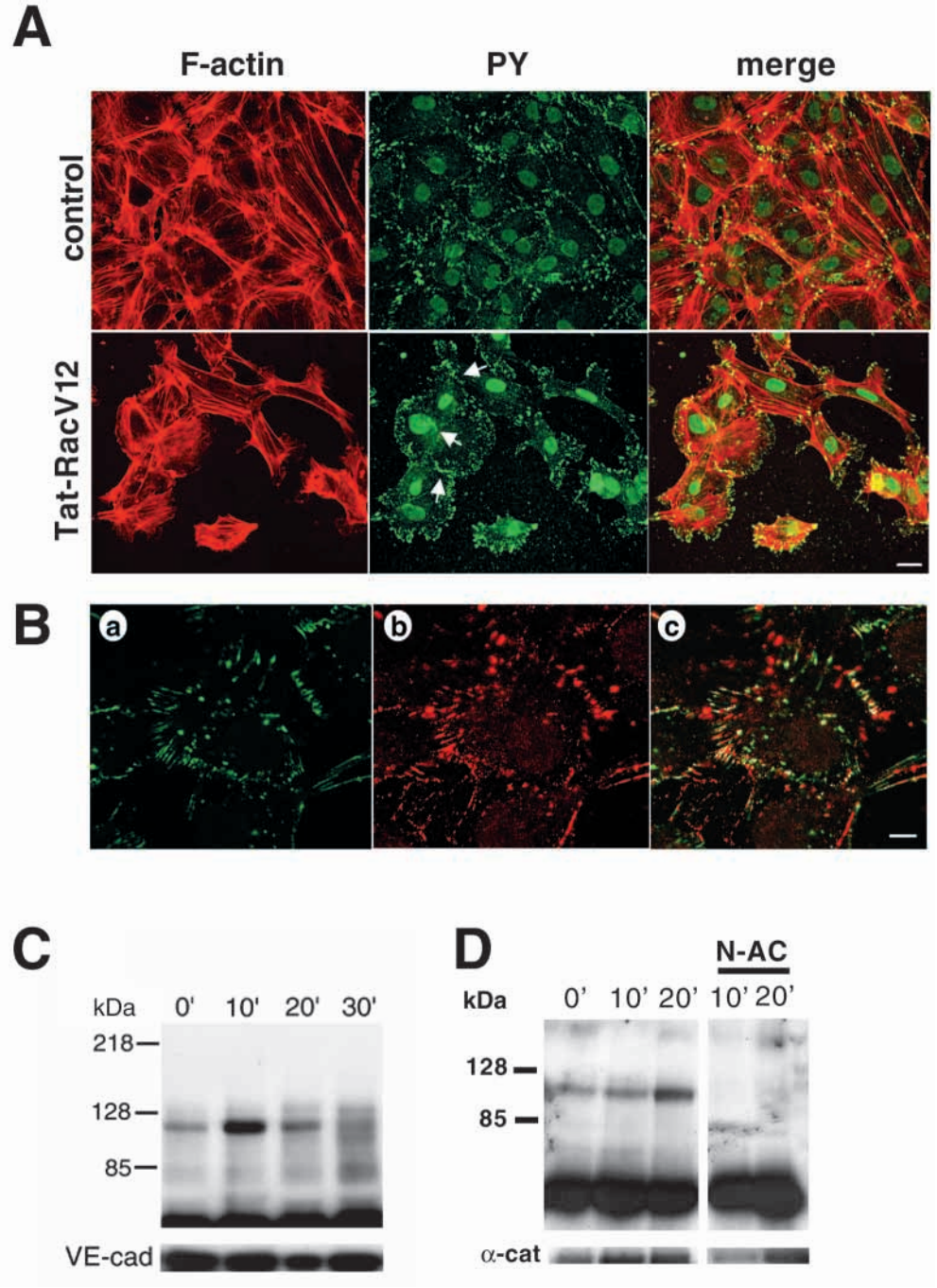

Fig. 6. Tat-RacV12 induces tyrosine phosphorylation of junctional proteins. (A) Primary HUVECs were treated with Tat-peptide (control) or Tat-RacV12 for 30 minutes, fixed and stained for F-actin (red) and phosphotyrosine (PY, green). Arrows indicate increased phosphorylation at cell borders and in between cells. Bar, 20 μm. (B) Primary HUVECs were treated with Tat-RacV12 for 30 minutes, fixed and stained for β-catenin (a) and phosphotyrosine (b). (c) shows the merged picture; colocalization as determined using specific software (identifying pixels with equal intensities of red and green) is indicated in white. Bar, 5 μm. (C) Primary HUVECs were treated with Tat-RacV12 for the various time periods indicated, after which VE-cadherin was immunoprecipitated. Western blots for phosphotyrosine (upper panel) and for VE-cadherin (lower panel, migrating at a molecular weight of approximately 130 kDa) are shown. VE-cadherin itself (130 kDa) is not phosphorylated upon Tat-RacV12 treatment. (D) Immunoprecipitation of α-catenin followed by western blotting for phosphotyrosine (upper panel) following Tat-RacV12 treatment for the time periods indicated with or without pretreatment with N-AC. The lower panel indicates blotting for α-catenin to control for equal loading.

(patho)physiological processes, such as inflammation, edema and tumor angiogenesis. This is because endothelial integrity is strictly dependent on VE-cadherin function; inhibitory antibodies induce a rapid and in some cases dramatic loss of cell-cell adhesion, inhibit angiogenesis and promote endothelial permeability (Corada et al., 1999; Corada et al., 2001; Hordijk et al., 1999; Liao et al., 2000). Moreover, VE-cadherin plays an important role in cell survival (Carmeliet et al., 1999), further underscoring its crucial and complex role in endothelial cell function. In the current work, we reveal a previously unrecognized signaling pathway that modulates endothelial cell-cell adhesion. We demonstrate that Rac, in conjunction with Rho-dependent contractility, reduces cadherin-based cell-cell adhesion and induces an increase in permeability. This effect is paralleled by and dependent on the synthesis of ROS and is accompanied by ROS-dependent tyrosine phosphorylation of the VE-cadherin complex.

The analysis of the role of Rac and Rho proteins in primary human endothelial cells was simplified significantly by using protein transduction, which has previously proven successful for a variety of proteins, including the small GTPase Rho in chicken osteclasts (Chellaiah et al., 2000) as well as in human eosinophils (Alblas et al., 2001).

The major finding of this study is that RacV12 induces loss of endothelial cell-cell adhesion through a pathway that involves the production of ROS and tyrosine phosphorylation

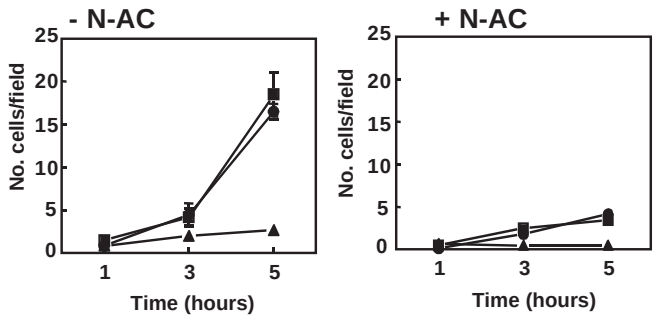

Fig. 7. Role for ROS in the migration of endothelial cells. pHUVEC, pretreated or not with 5 mM N-AC, were allowed to migrate for 5 hours towards 0% (▲), 5% (●) and 10% (■) (v/v) FCS in medium with or without the N-AC present during the assay, after which the cells were fixed, stained and counted by fluorescence microscopy as described in the Materials and Methods.

of the VE-cadherin complex. This negative effect of RacV12 on VE-cadherin function is in sharp contrast to its effect in epithelial cells where Rac and its exchange factor Tiam-1 promote E-cadherin function (Hordijk et al., 1997). Considering the fact that the basic principles of cadherin-mediated homotypic adhesion (composition of the cadherin-catenin complexes, regulation through the actin cytoskeleton) are very similar in epithelial cells and in endothelial cells, this result represents an intriguing discrepancy.

Although Rho-mediated cell contractility was required for RacV12-induced loss of cell-cell adhesion, Tat-RhoV14 showed limited effects on cell-cell junctions and monolayer permeability. These results may be explained by the fact that experiments were performed in the presence of 10% serum, a known activator of Rho, so that transduction with exogenous active Rho would have limited additional effects. Another explanation might be that Rho activity per se is not sufficient to open intercellular contacts and that RacV12, in addition to inducing Rho-dependent contractility, activates a complementary Rho-independent pathway that negatively controls endothelial cell-cell adhesion. This explanation is supported by our observation that scavenging ROS reduces Rac-mediated loss of cell-cell adhesion but does not affect the Rho-dependent formation of stress fibers. Recently, RacV12-mediated loss of cadherin-dependent adhesion was also described in keratinocytes (Braga et al., 2000). However, since the intracellular signaling underlying this effect was not studied, it is not clear whether similar pathways control cadherin function in keratinocytes and in endothelial cells. We have not been able to detect increased production of ROS by Tat-RacV12 in epithelial cell lines, and it needs to be investigated whether this pathway may be prevalent in primary cells. Finally, transduction of primary HUVEC with Tat-N17-Rac or Tat-N19-Rho proteins did not disturb VE-cadherin localization at cell-cell borders, indicating that, at least within the time frame of these experiments, Rac or Rho activity is not required to keep VE-cadherin at cellular junctions, which is in agreement with earlier studies (Braga et al., 1999).

The modulatory role of ROS in cell-cell adhesion is well known and is largely based on the addition of (high concentrations of) H_2O_2 to endothelial and epithelial cells, resulting in increased permeability, cellular injury and cell death (Lum and Roebuck, 2001). However, increasing evidence indicates that at low levels, ROS can also function as signaling molecules participating in the regulation of fundamental cellular processes such as cell growth, cell division and apoptosis (Finkel, 1999). A number of previous studies have suggested that Rac is involved in ROS production in endothelial cells, as it is in neutrophils, although it is not certain which type of ROS-generating enzyme is expressed in these cells. Rac-mediated ROS production has been implicated in TNF-α-induced endothelial apoptosis (Deshpande et al., 2000) and shear-stress induced tyrosine phosphorylation (Yeh et al., 1999) but to our knowledge, production of intracellular peroxide has so far not been implicated in the regulation of VE-cadherin function.

The mechanism of action of ROS-mediated loss of cadherin function is not established, but peroxide has been proposed to increase oxydation of crucial cysteine residues in tyrosine phosphatases (Rhee et al., 2000), leading to inactivation. This results in a net increase in tyrosine phosphorylation, which could lead to increased phosphorylation of the cadherin-catenin complex, an event that is generally associated with reduced cell-cell adhesion. Our data are in agreement with this hypothesis in that Tat-RacV12-induced ROS production is accompanied by ROS-dependent phosphorylation of α-catenin, which links the VE-cadherin complex to the cortical actin cytoskeleton. It is not known which phosphatase or kinase may be involved in this signaling, although it is intriguing that association of the SHP2 tyrosine phosphatase with VE-cadherin has recently been reported to be modulated by thrombin (Ukropec et al., 2000). Previous studies demonstrated a lack of α-catenin phosphorylation (Andriopoulou et al., 1999; Lampugnani et al., 1997). However, these studies were performed under different conditions and therefore difficult to compare. Although Tat-RacV12 induces delocalization of the VE-cadherin-catenin complex, we have not been able to detect Tat-RacV12-mediated dissociation of the complex, suggesting that the complex as it exists at the cell-cell junctions becomes disconnected from the cortical actin cytoskeleton, resulting in loss of adhesion.

The role of Rac-mediated ROS production in reducing VE-cadherin function may explain the endothelial damage that is associated with, for instance, ischemia-reperfusion injury, an effect that is known to be accompanied by the formation of ROS. Moreover, the modulation of VE-cadherin function through the intracellular levels of ROS appears to also be relevant for migration (Fig. 7), indicating that inhibition of ROS production may interfere with angiogenesis. This hypothesis is based on the fact that transient reduction of cadherin function is required for efficient endothelial cell migration (Cai et al., 1999; Liao et al., 2000). The general roles that intracellular ROS levels may play in endothelial integrity are further underscored by our finding that thrombin-mediated loss of cell-cell adhesion was also blocked following ROS scavenging (not shown), although we have not yet been able to detect thrombin-induced formation of ROS.

In conclusion, the Rac-ROS signaling pathway seems to be an important regulator of VE-cadherin function and cell-cell adhesion in primary human endothelial cells. Future research will focus on the type of oxidase that is relevant for endothelial cells and the pathway that leads to Rac-mediated

phosphorylation of the cadherin-complex and the concomitant loss of cadherin function.

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