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ICAM-3 Activation Modulates Cell-Cell Contacts of Human Bone Marrow Endothelial Cells

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Key Words
ICAM-3 · Endothelium · Reactive oxygen species · Resistance · Bone marrow

Abstract
The Ig-like cell adhesion molecule ICAM-3 is mainly expressed on human leukocytes and is involved in cell-cell interactions. Its expression on endothelium is observed during disorders such as Crohn's disease and in solid tumors. We found low but detectable expression of ICAM-3 on VE-cadherin-expressing cells from primary human bone marrow aspirates, i.e. endothelial cells, and on primary human endothelial cells from cord blood. Also, immortalized human umbilical cord endothelial cells and human bone marrow endothelial cells showed ICAM-3 expression. However, its function on human endothelium is not known. Surprisingly, activation of endothelial ICAM-3 by crosslinking with specific antibodies resulted in a drop in the electrical resistance of bone marrow endothelial monolayers. In line with this, immunocytochemical analysis showed a loss of endothelial cell-cell contacts after ICAM-3 crosslinking in HBMEC. Detailed biochemical analysis showed an association of moesin and in a later stage ezrin with ICAM-3 upon crosslinking in HBMEC. Moreover, ICAM-3 crosslinking induced the production of reactive oxygen species (ROS), which are known to be involved in the control of endothelial cell-cell contacts. In conclusion, we showed that ICAM-3 is expressed on human bone marrow endothelial cells and controls endothelial integrity via ROS-dependent signaling.

Introduction

The role for Ig-like cell adhesion molecules such as ICAM-1 (CD54) and VCAM-1 (CD106) in the process of leukocyte transendothelial migration is well established [1]. In addition, ICAM-2 (CD102) is involved in the migration of dendritic cells across the endothelium [2]. These proteins are localized to the apical side of the endothelium. A fourth member of this family, PECAM-1 (CD31), is found at endothelial junctions and is also involved in the passage of leukocytes across the endothelium [3]. In contrast, the Ig-like cell adhesion molecule ICAM-3 (CD50) seems to play no role in endothelium, but instead contributes to leukocyte-leukocyte interactions [4, 5].

ICAM-3 is constitutively expressed on human leukocytes, such as T and B lymphocytes, monocytes and neutrophils, and recognizes the β2-integrin leukocyte function associated molecule-1 (LFA-1) as a receptor, which is also the receptor for ICAM-1 and -2 [6]. It has been reported that ICAM-3 localizes to the rear of polarized leukocytes and clusters with the ezrin-radixin-moesin (ERM) proteins

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moesin and ezrin. These ERM proteins link ICAM-3 to the actin cytoskeleton [7–9]. Stimulation of ICAM-3 by crosslinking on T lymphocytes induces calcium fluxes in which protein tyrosine kinases $p56^{Lck}$ and $p59^{Fyn}$ are involved [5].

Although no prominent role for ICAM-3 on endothelium has been described, several studies have reported endothelial expression of ICAM-3 using histological detection methods [10–13]. Increased ICAM-3 expression on endothelium is observed in certain disorders, such as Crohn's disease and in solid tumors. It is suggested that ICAM-3 upregulation on endothelium in these diseases is induced by a mechanism similar to that known for ICAM-1 and VCAM-1 upregulation [12–14]. Some studies suggest a role for ICAM-3 on endothelium in early stages of angiogenesis, based on its expression in different stages of vascular differentiation of hemangiomas [10, 11].

In this study, we show that VE-cadherin-expressing cells from primary human bone marrow aspirates, i.e. endothelial cells, express ICAM-3. Moreover, expression of ICAM-3 on primary and immortalized human umbilical vein endothelial cells (HUVEC) and human bone marrow endothelial cells (HBMEC) is low, but detectable, and remains stable after treatment with different endothelial stimuli. Using the electrical cell-substrate impedance sensing technique, we show that ICAM-3 activation by crosslinking results in a drop in the electrical resistance of HBMEC. In addition, ICAM-3 activation generates reactive oxygen species (ROS) and is accompanied by the association of the ERM protein moesin with ICAM-3 in HBMEC. In conclusion, we show that ICAM-3 contributes to the control of the integrity of human bone marrow endothelial monolayers.

Materials and Methods

Reagents and Antibodies

Monoclonal antibodies (mAbs) to moesin and ezrin were from Transduction Laboratories (Becton Dickinson Company, Amsterdam, The Netherlands). β-Catenin and ezrin polyclonal antibody (Ab) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif., USA). Recombinant human interleukin (IL)-1β and tumor necrosis factor (TNF)-α were from PeproTech (Rocky Hill, N.J., USA); vascular endothelial growth factor $(VEGF)_{165}$ was from R&D Systems (Minneapolis, Minn., USA); Texas Red phalloidin, FITC-dextran 3000, Alexa 488- or Alexa 568-labeled goat-anti-mouse (GαM) Ig and Alexa 488-labeled goat-anti-rabbit Ig secondary Abs were from Molecular Probes (Leiden, The Netherlands). PE-labeled secondary Abs and mAb KS128 against ICAM-3 were from DAKO (Glostrup, Denmark). Pooled human serum, HSA, fibronectin (FN) and isotype mAbs IgG1, IgG1-FITC, IgG2a and IgG2a-PE (as control) were obtained from the CLB (Amsterdam, The Netherlands). FCS was from Gibco BRL (Life Technologies, Paisley, Scotland, UK). bFGF was from Boehringer Mannheim (Mannheim, Germany). mAb to ICAM-3 (HP2/19) and ICAM-3 conjugated with PE were purchased from Beckman Coulter (Mijdrecht, The Netherlands). mAb to ICAM-3 (CBR.IC3/1) and mAb to VE-cadherin, conjugated with FITC, were from Bender Medsystems (Vienna, Austria). Crosslinking studies were performed with $F(ab)_2$ fragments of GαM IgG from Jackson Immunoresearch (Baltimore, Md., USA). Oxygen radical scavengers N-acetyl-cysteine (N-AC) and tiron were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Cell Cultures

The HBMEC lines used (HBMEC-28, -33 and -60) have been described previously [15]. HBMEC and HUVEC lines 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 10% (v/v) pooled, heat-inactivated human serum, 10% (v/v) 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 confluence, the endothelial cells were passaged by treatment with trypsin-EDTA solution (Gibco BRL). The HBMEC-60 line was used in all experiments described, unless stated otherwise. In some experiments, HBMEC monolayers were pretreated with 10 ng/ml TNF-α overnight as indicated. All cell lines were cultured at 37°C in 5% CO_2.

Immunocytochemistry

HBMEC were cultured on FN-coated glass coverslips, fixed and immunostained as described elsewhere [16] with the polyclonal Ab to β-catenin (5 μg/ml), followed by staining with fluorescently labeled secondary Abs. F-actin was visualized by Texas Red phalloidin (1 U/ml). In some experiments, cells were pretreated with mAb to ICAM-3 (HP2/19 or IC3/1, 10 μg/ml) for 30 min, followed by 30 min of crosslinking with 10 μg/ml $F(ab)_2$ fragments of GαM IgG. 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.

Flow Cytometry

Following preincubations, the HBMEC or HUVEC were detached with 5 m*M* EDTA in calcium-free HEPES medium for 10 min at 37°C. After harvesting, the cells were incubated with the different mAbs (5 μg/ml) in PBS containing 0.5% BSA and 1 m*M* calcium for 30 min at 4°C and were washed with a 30-fold excess of ice-cold PBS-BSA. The cells were then incubated with PE-conjugated GαM IgG for 30 min at 4°C and washed. The relative fluorescence intensity was measured by flow cytometry (FACScan, Becton Dickinson).

Analysis of ROS

To measure intracellular ROS production, HBMEC were cultured on FN-coated glass coverslips and loaded with di-hydro-rhodamine-1,2,3 (30 μ*M*; Molecular Probes) for 60 min at 37°C. Adhesion molecules were subsequently crosslinked at 37°C as indicated in the figure legends. After 30 min, cells were washed twice, incubated for 1 min with trypsin at 37°C, collected on ice and again washed twice with ice-cold Ca^{2+}- and Mg^{2+}-containing PBS, and di-hydro-rhodamine-1,2,3 fluorescence was quantitated by flow cytometry in the FL-2 channel (λ_{EX} 488 nm, λ_{EM} 585 nm), as described elsewhere [17]. Intensity values are shown as the percentage increase relative to

the values at the start of the experiment. In some experiments, cells were pretreated for 18 h with a 5 m*M* concentration of the oxygen radical scavengers N-AC or tiron to prevent ROS-mediated signaling.

Immunoprecipitation and Western Blot Analysis

Cells were grown to confluence on 50-cm^2 FN-coated dishes, gently washed twice with ice-cold Ca^{2+}- and Mg^{2+}-containing PBS and lysed in 0.5 ml of lysis buffer (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% NP-40 and 0.5 m*M* orthovanadate with the addition of protease inhibitor cocktail tablets, Boehringer Mannheim). After 10 min on ice, cell lysates were collected and precleared for 30 min at 4°C with protein-G Sepharose (Pharmacia Biotech, Uppsala, Sweden; 15 µl for each sample of 0.5 ml). The supernatant, separated by centrifugation (14,000 *g,* 15 s at 4°C), was incubated with 15 µl of protein-G Sepharose, coated with 5 µg/ml ICAM-3 Ab (HP2/19), for 1 h 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 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to 0.45-µm nitrocellulose (Schleicher and Schuell Inc., USA), and the blots were incubated with the appropriate Abs (either moesin or ezrin diluted 1:2,000, or ICAM-3 diluted 1:1,000) in blocking buffer (1% low-fat milk in Ca^{2+}- and Mg^{2+}-containing PBS) overnight at 4°C, followed by incubation with rabbit-anti-mouse IgG-HRP (1:1,000, 1 h; DAKO) at room temperature. Between the various incubation steps, the blots were washed 3 times with Ca^{2+}- and Mg^{2+}-containing PBS with 0.05% Tween-20 and finally developed with an enhanced chemiluminescence detection system (Amersham).

Electric Cell-Substrate Impedance Sensing

Endothelial cells (HBMEC/HUVEC) were added at 100,000 cells to FN-coated electrode arrays (surface area 0.8 cm^2) and grown to confluence. Each array contains a 250-µm-diameter gold electrode and a much larger gold counter-electrode. After electrode check of the array and the electrical resistance of the endothelial monolayer under resting conditions, ICAM-3 was crosslinked as described above and the electrical resistance was continuously monitored at 37°C in 5% CO_2 with the ECIS-Model-100 Controller from BioPhysics, Inc. (Troy, N.Y., USA). Data were collected for a period of 2 h, and changes in the resistance of different endothelial monolayers were analyzed.

Permeability Assay

Permeability of HBMEC monolayers, cultured on 5-µm-pore, 6.5-mm Transwell filters (Costar, Cambridge, Mass., USA), was assayed using FITC-labeled dextran 3000 as described elsewhere [17]. The monolayers were pretreated with Abs (10 µg/ml) for 30 min as described above. Crosslinking Ab was present during the permeability assay. After the assay, filters were washed with ice-cold Ca^{2+}- and Mg^{2+}-containing PBS, then fixed with 2% paraformaldehyde and 1% Triton X-100-containing PBS and stained with Texas Red phalloidin to inspect the HBMEC monolayer by confocal laser scanning microscopy.

Statistics

Student's t test for paired samples (two-tailed) was used for statistical analysis. Student's t test for independent samples was used where indicated.

Results

Analysis of freshly isolated venous endothelial cells from umbilical cord (pHUVEC) by flow cytometry revealed low but detectable expression of the Ig-like superfamily member ICAM-3 (CD50) (fig. 1A). To study ICAM-3 expression from primary HBMEC, bone marrow aspirates were analyzed for ICAM-3 expression. Although the total number of endothelial cells (defined as VE-cadherin-positive cells) in primary bone marrow aspirates is low (0.16%), flow cytometry analysis showed that an average of 49% of the VE-cadherin-positive cells also stained positive for ICAM-3 (fig. 1B). Western blot analysis of ICAM-3 protein on whole-cell lysates from immortalized umbilical cord (HUVEC) and bone marrow (HBMEC) confirmed the expression of the protein and additionally showed that the expression of ICAM-3 in HBMEC was more pronounced than in HUVEC (fig. 1C). Thus, ICAM-3 is expressed on primary and immortalized endothelium from umbilical cord and bone marrow.

Pretreatment of HBMEC (fig. 2) or pHUVEC (data not shown) with the inflammatory mediators IL-1β, TNF-α or both induced upregulation of the surface expression of ICAM-1 (CD54) and VCAM-1 (CD106), whereas the expression of PECAM-1 (CD31) and VE-cadherin (CD144) was not affected (fig. 2). The expression of ICAM-3 did not significantly change under these conditions on either endothelial cell type. ICAM-2 (CD102) was slightly downregulated upon IL-1β and TNF-α treatment (fig. 2). Because some reports suggest a role for ICAM-3 in angiogenesis, the endothelial cells were also stimulated with VEGF. However, the expression of none of the tested adhesion molecules was affected (fig. 2). Thus, because the expression of ICAM-3 is not affected by inflammatory stimuli, in contrast to its family members ICAM-1 and VCAM-1, these results suggest that ICAM-3 may be involved in processes such as constitutive lymphocyte trafficking.

Biochemical analysis of ICAM-3 immunoprecipitates from HBMEC revealed a 120-kD band, corresponding to the size of ICAM-3 (fig. 3A). An unidentified protein coimmunoprecipitated at approximately 97 kD. Studies in T lymphocytes show that the ERM protein moesin binds to ICAM-3, linking this Ig-like adhesion molecule to the actin cytoskeleton [9]. Activation of ICAM-3 by crosslinking on HBMEC monolayers resulted in an increased interaction of ICAM-3 with moesin, but not ezrin, after 5–15 min. After 30 min of crosslinking on HBMEC monolayers, the association with moesin was lost but the binding to ezrin was increased (fig. 3B). Incubation of the cells

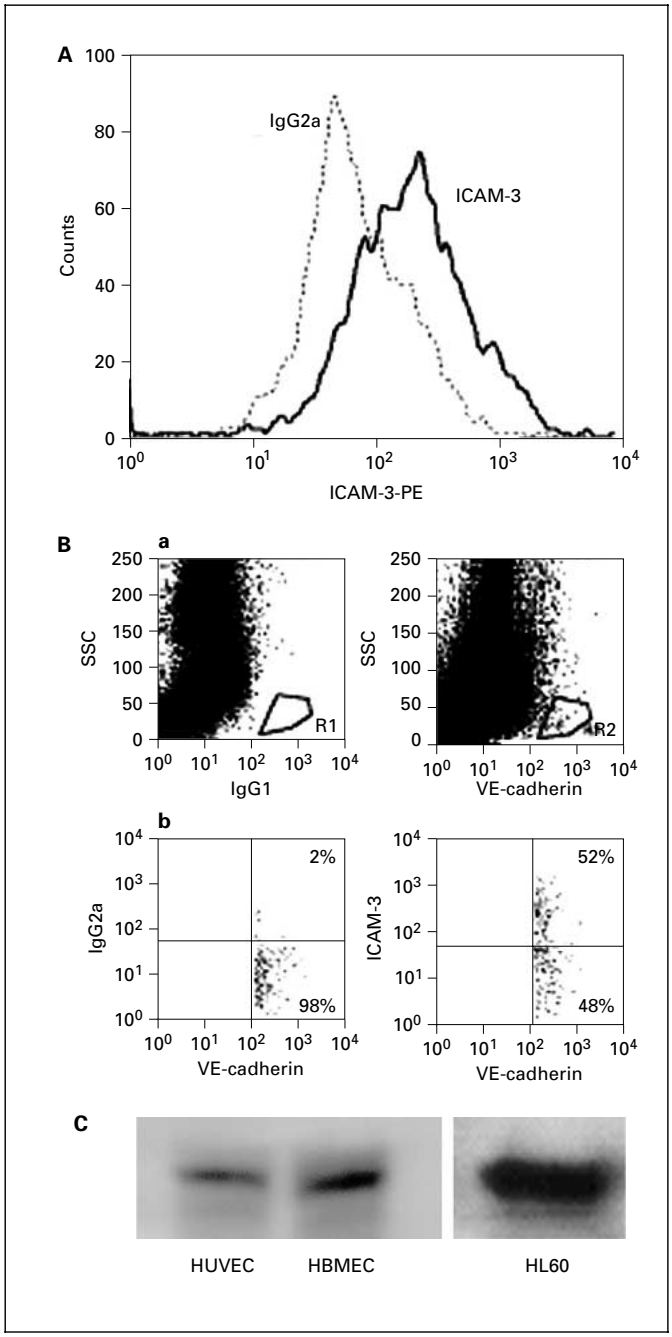

Fig. 1. Expression of ICAM-3 on human endothelial cells. **A** Flow cytometry analysis of pHUVEC, showing expression of ICAM-3 (solid line). The dotted line indicates the signal obtained with the isotype IgG2a Ab. **B** Human bone marrow aspirates showed ICAM-3 expression on 52% of VE-cadherin-positive cells. The mean of three independent experiments showed 49% VE-cadherin-positive cells. **a** Scatter plot of flow cytometry analysis showing cells from the aspirate which were incubated with IgG1-FITC or VE-cadherin-FITC. 0.16% of all cells (R1) stained positive for VE-cadherin. Results are data of one experiment, representative of 3 independent experiments. **b** 52% of VE-cadherin-positive cells stained positive for ICAM-3. Data in **B** are representative of 3 independent analyses. **C** Western blot analysis of total cell lysates with ICAM-3 Ab showed a band at approximately 120 kD, corresponding to the molecular weight of ICAM-3. 2×10^6 endothelial cells (HBMEC or HUVEC) and 0.5×10^6 HL60 cells, as a positive control, were loaded per lane. Results are representative of four independent experiments.

with IgG2a Abs as a control induced no changes in the association of ICAM-3 with either moesin or ezrin. These findings indicate that, upon activation of ICAM-3 in HBMEC, first moesin and later ezrin interacts with the intracellular tail of clustered ICAM-3, as in T lymphocytes. This interaction might transmit intracellular signaling into the endothelial cells.

Because moesin and ezrin can link activated ICAM-3 to the actin cytoskeleton, and the actin cytoskeleton controls cell-cell adhesion, we studied the effects of ICAM-3 crosslinking on the integrity of the endothelial monolayers. Therefore, we used the electric cell-substrate impedance sensing technique to measure the electrical resistance of the endothelial monolayers following various treatments. The basal resistance of the HBMEC monolayer was significantly higher than that of the HUVEC line monolayers and the pHUVEC monolayers (8.4 ± 0.3 vs. 7.1 ± 0.2 and 7.2 ± 0.3 kΩ, respectively) (fig. 4A). Other HBMEC lines (HBMEC-28 and -33) were also tested and showed similar results to the HBMEC-60 line (data not shown). Expression levels of the endothelial junction proteins VE-cadherin and PECAM-1, which are involved in the maintenance of the barrier function, were similar in both types of endothelial cells, as determined by flow cytometry (data not shown).

Surprisingly, activation of ICAM-3 by Ab-mediated crosslinking decreased the electrical resistance of the TNF-α-pretreated HBMEC monolayer within 60 min (fig. 4B). The resistance of the HBMEC monolayer was restored after approximately 4–5 h, although occasionally the resistance of the monolayer was restored already after 2 h of ICAM-3 activation. Control levels of the HBMEC monolayers sometimes showed an increase in the resistance. However, this increase was not consistent throughout different experiments, whereas the drop in resistance of the HBMEC monolayer after ICAM-3 crosslinking was. To further quantify the effect of ICAM-3 activation, we analyzed the ICAM-3-induced decrease in resistance

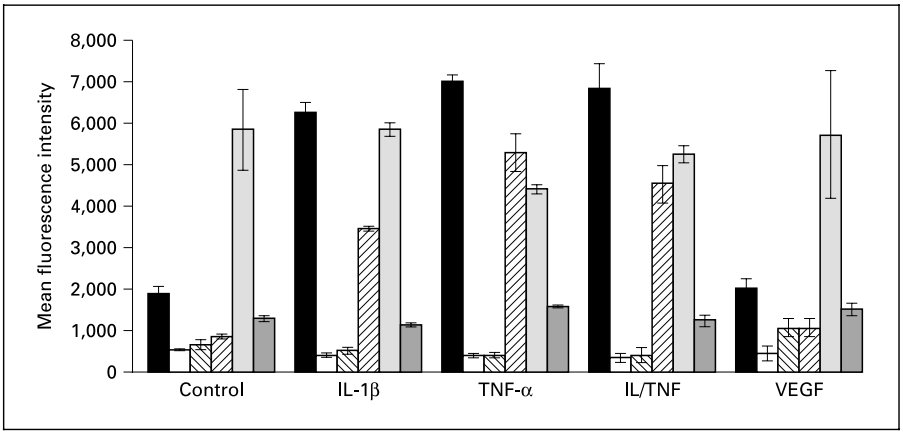

Fig. 2. Expression of adhesion molecules on HBMEC. HBMEC were cultured on FN-coated 8-cm^2 dishes and stimulated for 6 h with IL-1β (10 ng/ml), TNF-α (10 ng/ml), both or VEGF (100 ng/ml). Expression analysis was performed with flow cytometry. ICAM-1 and VCAM-1 showed increased expression upon stimulation with IL-1β, TNF-α or both, whereas ICAM-2 showed a slight decrease in expression upon treatment with these stimuli. PECAM-1, VE-cadherin and ICAM-3 showed no change in expression levels. Stimulation with VEGF did not have any effect on the expression levels of any of the tested adhesion molecules. Closed bar: ICAM-1; open bar: ICAM-2; hatched bar left: ICAM-3; hatched bar right: VCAM-1; light grey bar: PECAM-1; dark grey bar: VE-cadherin. Data are mean ± SD of 4 independent experiments.

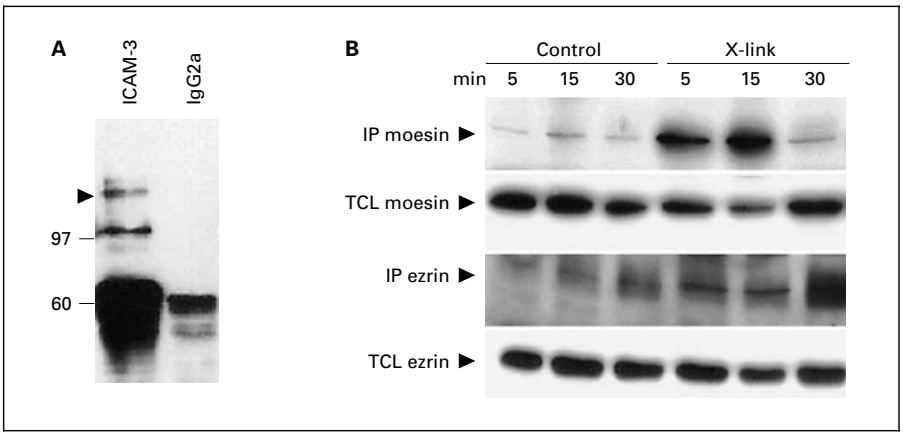

Fig. 3. Interaction of ICAM-3 with moesin and ezrin. **A** HBMEC were cultured on FN-coated 50-cm^2 dishes and grown to confluence. Cells were lysed and ICAM-3 was immunoprecipitated from the lysate as described in Materials and Methods. Isotype control IgG2a was taken as a control sample. The immunoblot was incubated with Ab against ICAM-3. The arrowhead indicates ICAM-3 protein of approximately 120 kD in untreated HBMEC (2×10^6 cells). An unidentified protein coimmunoprecipitated at approximately 97 kD. The 60-kD band indicates the heavy chain of the precipitating Ab. **B** HBMEC were cultured and treated as described above. ICAM-3 was activated (X-link) for 5, 15 or 30 min as indicated. Control represents HBMEC incubated with mAb against isotype IgG2a and subsequently crosslinked for 5, 15 or 30 min as indicated. ICAM-3 was immunoprecipitated (IP) and samples were blotted with mAbs against moesin or ezrin, as indicated. The association with ICAM-3 of moesin and, after 30 min, of ezrin, was induced by crosslinking. Lower panels show protein expression of moesin and ezrin in total cell lysates (TCL), to show that each immunoprecipitate contained equal amounts of protein. Data are representative of 2 independent experiments.

of the HBMEC and HUVEC monolayer after 60 min. The results revealed that ICAM-3 activation on HBMEC significantly reduced the resistance of the monolayer, whereas ICAM-3 activation on HUVEC did not (fig. 4C). Monolayers of three HBMEC lines (HBMEC-60, -28 and -33) were tested, and all three cell lines responded to ICAM-3 activation with a significant decline in monolayer resistance at 60 min. Together, these results indicate that the activation of ICAM-3 on HBMEC, but not on HUVEC, negatively regulates cell-cell junctions, resulting in a

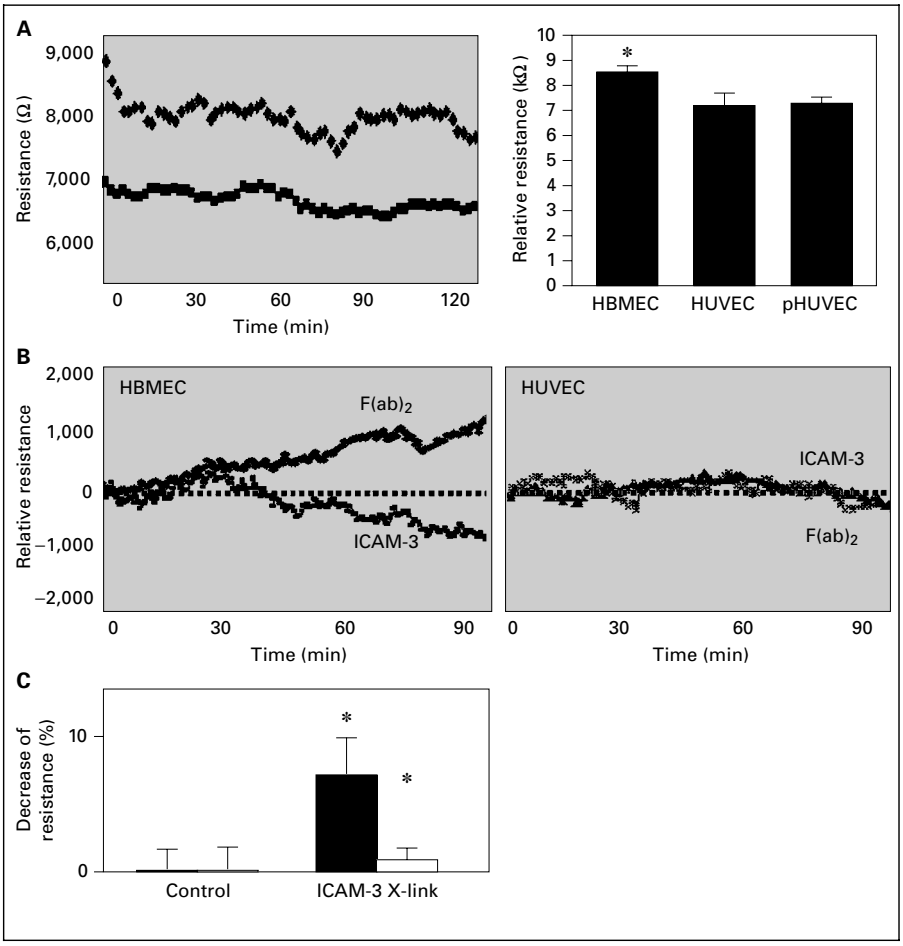

Fig. 4. ICAM-3 activation decreases the resistance of HBMEC monolayers, but not of HUVEC monolayers. **A** Endothelial cells were plated on FN-coated electrode arrays in equal amounts and cultured to confluence. The left panel shows a representative recording of the basal resistance of a HUVEC line monolayer (squares) and HBMEC (triangles) monitored over 2 h. The right panel shows the average of the basal resistance of HBMEC and HUVEC monolayers at 60 min and indicates that the resistance of the HBMEC monolayer is significantly higher than that of HUVEC monolayers. Data are means ± SD of 3 independent experiments. Each experiment was carried out in quadruplicate. *$p < 0.05$. **B** The left panel shows the electrical resistance of HBMEC monolayers, which were plated on FN-coated electrode arrays and cultured to confluence. The electrical resistance of the endothelial monolayers was monitored for 2 h. ICAM-3 was activated at time point 0 min and induced a drop in the electrical resistance of the monolayer within 60 min. The lower line indicates ICAM-3 crosslinking on the HBMEC monolayer, while the upper line shows the HBMEC monolayer, incubated with only $F(ab)_2$ Ab, as indicated. Each experiment was carried out in quadruplicate and done at least three times for HBMEC-60; HBMEC-28 and -33 were tested twice. The right panel shows the electrical resistance of HUVEC monolayers, treated as indicated. Each experiment was carried out in quadruplicate and done at least three times for primary and immortalized HUVEC. **C** At 60 min after ICAM-3 activation, the changes in HBMEC and HUVEC monolayer resistance were quantified, which showed that ICAM-3 activation significantly reduced the resistance of HBMEC (filled bars) but not of HUVEC (open bars). *$p < 0.05$. X-link = Crosslinking.

decrease in resistance of the human bone marrow endothelial monolayers.

In line with the latter observation, detailed confocal laser scanning microscopy analysis of cell-cell junctions of HBMEC after ICAM-3 activation showed a loss of cell-cell contacts, which explains the drop in resistance of the HBMEC monolayers after ICAM-3 activation (fig. 5A). Staining for the junctional protein β-catenin showed an altered distribution at sites of gap formation. Real-time analysis of ICAM-3 activation on HBMEC monolayers by phase-contrast microscopy indicated that after 30–60 min of ICAM-3 activation, loss of cell-cell contacts is induced (fig. 5B; also video 1 and control in video 2, see Supplemental Video Files below). Additional confocal microsco-

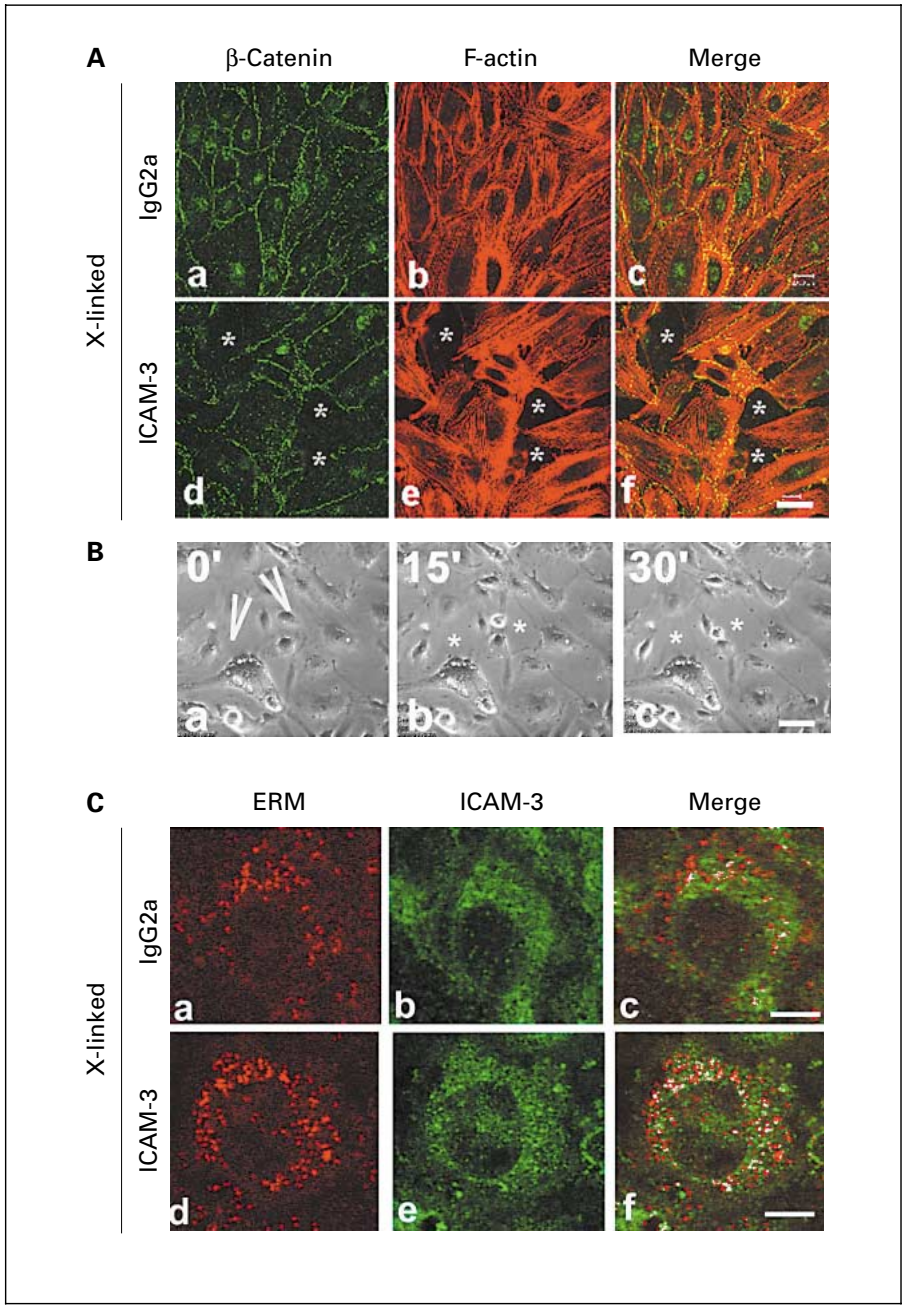

Fig. 5. Effect of ICAM-3 crosslinking on cell-cell contacts of HBMEC. **A** HBMEC were grown to confluence on FN-coated glass coverslips, stimulated with TNF-α overnight and treated for 60 min as indicated, fixed and permeabilized and subsequently stained for β-catenin and F-actin as described in Materials and Methods. The images show β-catenin in green (**a**, **d**) and F-actin in red (**b**, **e**), while colocalization appears in yellow (**c**, **f**). Incubation of endothelium with isotype control IgG2a Ab showed normal β-catenin localization at cell-cell junctions (**a**). F-actin staining showed a normal pattern of stress fibers and no loss of cell-cell contact (**b**). Merge shows combined images (**c**). Crosslinking of ICAM-3 (X-linked) induced loss of endothelial cell-cell contacts (indicated by the asterisks) in the HBMEC monolayer (**e**), with loss of discrete β-catenin localization at sites of absent cell-cell contacts (**d**). Merge shows combined images (**f**). Bar = 20 μm. **B** Real-time analysis by phase-contrast microscopy showed that ICAM-3 activation induced a loss of HBMEC cell-cell contacts within 30 min. Still images are taken from a time-lapse video. Crosslinking Ab was added at 0 s (**a**). Open arrowheads represent the sites where loss of cell-cell contact will appear. Gaps (asterisks) start to appear at approximately 15 min (**b**). Endothelial cell-cell contact is lost at approximately 30 min (**c**). (See also videos 1 and 2, the latter showing crosslinking of IgG2a mAb; for details, see Supplemental Video Files below). Bar = 50 μm. **C** HBMEC were cultured and treated as described in **A**. To stain ERM proteins, a polyclonal Ab against ezrin was used, which recognized all three ERM proteins. Images show ERM protein staining in red (**a**, **d**) and ICAM-3 staining in green (**b**, **e**), while colocalization is indicated in white (**c**, **f**). Incubation of endothelium with isotype control IgG2a mAb showed almost no induction of colocalization between the ERM proteins and ICAM-3 (**c**). The ERM proteins showed a punctate distribution over the apical cell surface (**a**), in a similar fashion to ICAM-3 (**b**). Crosslinking of ICAM-3 (X-linked) induced a loss of endothelial cell-cell contacts of the HBMEC monolayer (as shown in **A**), with increased colocalization between the ERM proteins and ICAM-3 (**f**). Bar = 5 μm.

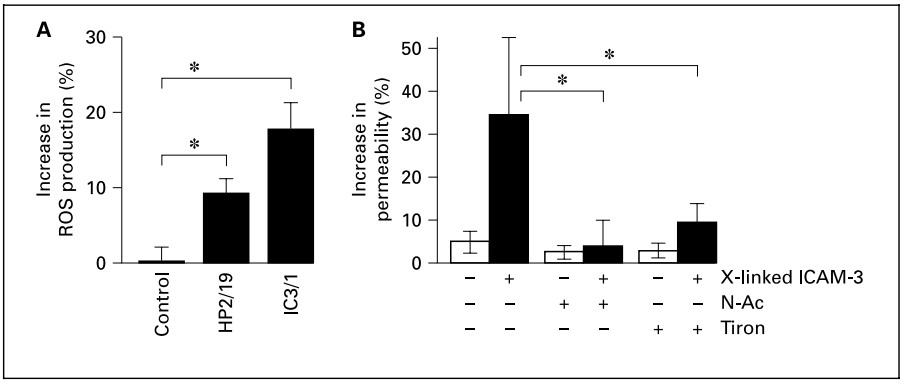

Fig. 6. Role of ROS in endothelial ICAM-3 signaling. **A** HBMEC were grown to confluence and loaded with di-hydro-rhodamine-1,2,3. Subsequently, the cells were treated as indicated, and after 30 min, the production of ROS was analyzed by flow cytometry as described in Materials and Methods. Crosslinking of ICAM-3 with mAb HP2/19 or CBR.IC3/1 (both 10 μg/ml) showed a small but significant increase in ROS production compared to control levels. Control levels represent crosslinking of IgG2a mAb. Data are means ± SD of 3 independent experiments. * $p < 0.05$. **B** Scavenging ROS results in decreased loss of cell-cell contacts. HBMEC were cultured on FN-coated Transwell filters and incubated overnight with medium and the oxygen radical scavengers N-AC or tiron at 37°C, as indicated. Subsequently, ICAM-3 (filled bars) or IgG2a, as a control (open bars), were crosslinked as described in Materials and Methods (X-linked ICAM-3 with mAb HP2/19), and FITC-labeled dextran 3000 was added to the upper compartment of the Transwell system. After 3 h, the amount of fluorescence in the lower compartment was calculated as the percentage increase compared to control levels. Control levels represent basal leakage of the endothelial monolayer incubated with medium alone. Data are means ± SD of 3 independent experiments. * $p < 0.05$.

py analysis after ICAM-3 activation showed that the ERM proteins moesin and ezrin are localized at the apical side of the endothelial cell surface (fig. 5C). Moreover, after 60 min of ICAM-3 activation, colocalization is observed between ICAM-3 and a fraction of the ERM proteins. This is probably due to the interaction of the ERM proteins with other adhesion molecules, such as ICAM-1 and CD44, which are also present on the apical side of the endothelial cells [7]. The polyclonal Ab used in these localization studies recognized all three ERM protein members, ezrin, radixin and moesin. Therefore, we were not able to discriminate between moesin and ezrin localization in these assays.

We and others have shown that members of the Ig-like superfamily such as VCAM-1 and ICAM-1 can act as signaling receptors [17–20]. In addition, we have shown that upon activation, VCAM-1 generates ROS and subsequently induces loss of endothelial cell-cell contacts [17]. Therefore, we studied the ability of ICAM-3 to generate ROS in HBMEC after activation by using the di-hydro-rhodamine-1,2,3 assay, as described in Materials and Methods. The results of this assay, performed with two independent Abs, indicate that crosslinking of ICAM-3 induced a small but significant increase in the production of intracellular ROS in HBMEC after 30 min compared to control levels (fig. 6A). Crosslinking of ICAM-2, also expressed on the apical membrane of the endothelial cells, did not induce detectable amounts of ROS (data not shown).

To study the involvement of ROS in ICAM-3-induced loss of endothelial cell-cell contacts, i.e. increased permeability, HBMEC were preincubated overnight with the oxygen radical scavengers N-AC or tiron. As a result, the scavengers significantly prevented the ICAM-3-induced increase in permeability, suggesting that ROS mediate ICAM-3-induced loss of endothelial integrity in HBMEC (fig. 6B). The inhibitors N-AC and tiron had no significant effect on the basal resistance of the HBMEC monolayer.

Discussion

Although the majority of the current literature states that ICAM-3 is not expressed by human endothelium, some studies indicate that ICAM-3 is expressed on human endothelial cells in certain disorders, such as Crohn's disease and cancer [6, 7, 9]. We found that ICAM-3 is expressed on primary human endothelial cells isolated from normal human bone marrow and umbilical cord, although the basal expression is low and expression is not upregulated by inflammatory stimuli, such as IL-1β or TNF-α.

Because endothelial ICAM-3 is expressed at low levels and is not upregulated by inflammatory stimuli, it is probably not involved in leukocyte transendothelial migration. In line with this, we were unable to inhibit stromal-cell derived factor-1 (SDF-1)-induced migration of HL60 cells across endothelium with ICAM-3-blocking Abs in a Transwell assay [van Buul, unpubl. observation].

Surprisingly, activation of ICAM-3 on immortalized human endothelial cells derived from bone marrow (HBMEC) induces a decline of the basal electrical resistance of these monolayers, whereas the resistance of endothelial monolayers from human umbilical cord blood (HUVEC) remains unaltered. The fact that the basal resistance of HBMEC monolayers is higher than that of HUVEC indicates a different regulation of the cell-cell junctions, although we found no differences in expression levels of the junctional adhesion molecules VE-cadherin and PECAM-1. These results show that endothelial cells derived from different origins are also different in their ability to regulate their endothelial junctions and barrier function.

The fact that ICAM-3 is expressed on HBMEC as well as on HUVEC, whereas ICAM-3 activation only has an effect on HBMEC monolayer resistance, is remarkable, although Western blot data already showed that ICAM-3 expression in HUVEC is lower than in HBMEC. This difference in expression level might explain the different effects of ICAM-3 activation on the monolayer resistance of HBMEC and HUVEC. Some studies suggest a role for ICAM-3 in early angiogenesis [10, 11]. However, treating endothelial cells with recombinant VEGF, which is known to be involved in the process of vascular angiogenesis [21], did not change the expression levels of ICAM-3. In addition, because motile endothelial cells are characteristic for angiogenesis, we checked whether ICAM-3 activation induces the motility of HBMEC using a Transwell assay. The results revealed that ICAM-3 activation did not increase endothelial motility, whereas fetal calf serum, as a positive control, did [van Buul, unpubl. observation]. Based on these data, we conclude that it is unlikely that ICAM-3 on endothelial cells is involved in cell motility. However, whether ICAM-3 plays a role in the modulation of endothelial integrity during an angiogenic response remains an open question.

The interaction of ICAM-3 with the cytoskeletal adapter proteins moesin and ezrin is not restricted to leukocytes only, but appears to occur also in HBMEC. Moreover, activation of ICAM-3 on HBMEC increased the association of first moesin and later ezrin with ICAM-3. Since the ERM proteins are known as adapter proteins linking apical surface proteins to the actin cytoskeleton, the increased association might induce cytoskeletal changes. ERM proteins have also been reported to activate signaling via the small GTPase RhoA, which induces the formation of actin stress fibers and increased cell contractility [22]. Conversely, Shaw et al. [23] reported that RhoA activity is necessary and sufficient to phosphorylate radixin and moesin. These data, together with the results presented in this paper, might suggest that ICAM-3 activation induces changes in the actin cytoskeleton through the ERM proteins in a RhoA-dependent manner. In addition, our results show that ICAM-3 activation decreases the resistance of HBMEC monolayers, probably by modulating the lateral cell-cell junctions, which results in a loss of barrier function. Pujuguet et al. [24] recently reported that ezrin is capable of modulating E-cadherin-based cell-cell contacts in epithelial cells. Whether ezrin and possibly moesin modulate the VE-cadherin complex in endothelial cells will require future study.

The hypothesis that ICAM-3 expression on endothelium is involved in tumor progression is in line with experiments that show ROS generation after ICAM-3 activation. The fact that ICAM-3 expression on endothelium is found on solid tumors and that oxygen radicals are elevated in tumor environments and are involved in tumor angiogenesis might indicate that ICAM-3 on endothelium contributes to tumorigenesis, although it remains unknown how ICAM-3 becomes activated under these conditions. Additional experiments show that not all apically expressed adhesion molecules induce ROS production. Although it is reported that ICAM-1 and VCAM-1 are involved in the production of endothelial ROS, we observed that ICAM-2 lacks the ability to produce ROS, underscoring the specificity of this response [17, 19, 25]. Our previous work suggests that VCAM-1-mediated production of ROS is involved in transendothelial migration [17]. However, ICAM-3-mediated ROS production seems to play no role in transendothelial migration. Therefore, we hypothesize that although the amount of ROS production mediated by ICAM-3 and VCAM-1 might be comparable, the outcome of their activation could be different.

In conclusion, we have shown that ICAM-3 negatively regulates endothelial cell-cell contacts of human bone marrow endothelium. Together with the knowledge that ICAM-3 is upregulated on endothelium in disorders such as Crohn's disease and tumors, these data suggest that ICAM-3 is involved in the dissociation of endothelial cells during the formation of new blood vessels.

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Supplemental Video Files

To view supplemental videos 1 and 2, please refer to http://www.karger.com/doi/10.1159/000076126

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