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Endothelial Cell Cortactin Coordinates Intercellular Adhesion Molecule-1 Clustering and Actin Cytoskeleton Remodeling during Polymorphonuclear Leukocyte Adhesion and Transmigration


Endothelial cell ICAM-1 interacts with leukocyte β2 integrins to mediate adhesion and transmit outside-in signals that facilitate leukocyte transmigration. ICAM-1 redistribution and clustering appear necessary for leukocyte transmigration, but the mechanisms controlling ICAM-1 redistribution and clustering have not been identified. We recently reported that Src kinase phosphorylation of endothelial cortactin regulates polymorphonuclear cell (PMN) transmigration. In this study, we tested the hypotheses that the Src family kinase-cortactin pathway mediates association of ICAM-1 with the actin cytoskeleton and that this association is required for ICAM-1 clustering and leukocyte transmigration. Cross-linking ICAM-1 induced cytoskeletal remodeling and a decrease in ICAM-1 lateral mobility, as assessed by fluorescence recovery after photobleaching. Cytoskeletal remodeling after ICAM-1 cross-linking was reduced by knockdown of cortactin by small interfering RNA, by expression of a cortactin mutant deficient in Src phosphorylation sites (cortactin3F), and by the Src kinase inhibitor PP2. Pretreatment of cytokine-activated human endothelial monolayers with cortactin small interfering RNA significantly decreased both actin and ICAM-1 clustering around adherent PMN and the formation of actin-ICAM-1 clusters required for PMN transmigration. Our data suggest a model in which tyrosine phosphorylation of cortactin dynamically links ICAM-1 to the actin cytoskeleton, enabling ICAM-1 to form clusters and facilitate leukocyte transmigration.

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was initially described as a regulator of dynamic cytoskeletal remodeling involved in cell mobility. Recent reports have suggested that cortactin recruits and binds to ICAM-1 and E-selectin in endothelial cells, and subsequently becomes phosphorylated by Src family kinases upon adhesion molecule engagement (14). Our lab has recently used live cell fluorescence imaging microscopy to show that endothelial cortactin redistributes and clusters around PMN transmigrating through TNF-α-activated HUVEC monolayers (15). Moreover, knockdown of cortactin by protein small interfering RNA (siRNA) or interruption of Src kinase-dependent cortactin phosphorylation in endothelial cells significantly impairs PMN transmigration at cell-cell borders (15). The mechanism by which ICAM-1 interacts with Src and cortactin during leukocyte TEM under flow has not been elucidated.

Based on the facts that both ICAM-1 and cortactin are important for leukocyte TEM and that cortactin regulates cytoskeletal remodeling, we hypothesized that cortactin is a critical linker protein that integrates ICAM-1 engagement to cytoskeletal remodeling and leukocyte TEM. In this study, we characterize the role of endothelial cortactin in ICAM-1-mediated cytoskeletal remodeling, and show that cortactin plays a critical role in ICAM-1 and F-actin clustering during the process of PMN transmigration.

Materials and Methods
Materials

Human rTNF-α was from PeproTech. PP2 was from Calbiochem. Phalloidin conjugated with Alexa Fluor 546 was from Molecular Probes. Non-blocking mAb to ICAM-1 (CL203.4) was conjugated with Alexa Fluor 568 (Molecular Probes). Cortactin mAb (4F11) was purchased from Upstate Biotechnology. A predesigned siRNA oligonucleotide targeting human cortactin mRNA and a scrambled version of this sequence (Ambion) were transfected into endothelial cells, as described previously (15).

Endothelial cell culture and leukocyte isolation

HUVEC were isolated, pooled, and cultured, as described (2). Human papillomavirus-immortalized HUVEC (16, 17) (iHUVEC) stably expressing wild-type human ICAM-1 or a tailless ICAM-1 mutant with cytoplasmic domain deletion (passages 7–10) were prepared and maintained, as previously described (3). Endothelial cells were seeded on glass coverslips precoated overnight with fibronectin (5 μg/ml; Sigma-Aldrich). Human PMN (>95% pure) were isolated from sodium citrate-anticoagulated whole blood drawn from healthy volunteers, as described (2). Blood was drawn and handled according to Brigham and Women’s Hospital Institutional Review Board-approved protocols for protection of human subjects, and all volunteer subjects gave informed consent.

Virus production and infection

Adenovirus encoding GFP-fused β-actin was produced in our lab by transfecting β-actin-GFP plasmid (18) into an adenovirus expression vector system using an previously detailed approach (19). Retroviruses encoding murine cortactin-GFP or cortactin3F-GFP (tyrosine 421, 466, and 482 residues mutated to phenylalanine (13)) were produced and HUVEC infected with these viruses, as previously described (15).

ICAM-1 cross-linking

Confluent endothelial monolayers were incubated with ICAM-1 mAb (HU8/3 (20), 10 μg/ml) for 30 min at 37°C. After washing three times in Dulbecco’s PBS (DPBS”), monolayers were incubated with 5 μg/ml rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 10 min at 37°C, followed by three washes in DPBS”. HUVEC were then used immediately in experiments.

Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed using a laser-scanning confocal microscope (LSM META 510 system; Zeiss). A circular area 1.7 μm in diameter was photobleached and then periodically scanned after the photobleach to monitor fluorescence recovery (Fig. 2, A and B). ICAM-1-GFP diffusion coefficient (Fig. 2C) was measured on confluent ICAM-1-GFP or tailless ICAM-1-GFP iHUVEC using an Ultima interactive laser cytometer (Meridian Instruments). The Gaussian beam radius was 1 μm, and excitation and emission wavelengths were 488 and 530 ± 15 nm, respectively. Photobleaching power at the sample was ~0.5 mW, and the bleach time was 75 ms. The laser beam was focused on an area of GFP fluorescence on the apical endothelial surface. GFP fluorescence in the illuminated area was measured three times before the photobleach (prebleach) and periodically after photobleach. Diffusion coefficients were calculated, as described (21, 22).

PMN adhesion and transmigration assay

The live cell fluorescence microscopy flow model has been described previously (2, 3). Confluent HUVEC monolayers were activated with TNF-α (25 ng/ml, 4 h), and then ICAM-1 was immunolabeled with Alexa 568-tagged anti-ICAM-1 mAb CL203.4 (10 min, 0.7 μg/ml). PMN (1 × 106/ml) suspended in flow buffer (DPBS containing 0.1% human serum albumin, 0.75 mmol/L Ca2+ and Mg2+) were drawn across HUVEC at 1.0 dyne/cm² for 1 min, followed by flow buffer alone for 10 min. Where noted, HUVEC monolayers were fixed for 8 min at room temperature with 10% buffered formalin (Sigma-Aldrich) after 2 or 5 min of flow and subjected to confocal microscopy.

FIGURE 1. PMN transmigrate through actin ring-like structures that colocalize with clustered endothelial cell ICAM-1. A, Confluent HUVEC monolayers transduced with actin-GFP adenovirus were activated with TNF-α for 4 h. These monolayers were prelabeled with Alexa 568-conjugated ICAM-1 mAb (0.7 μg/coverslip) and inserted into the in vitro flow chamber, and PMN were drawn through the chamber for 5 min before the experiments were terminated by fixation (10% formalin-buffered saline). Distribution of actin-GFP and ICAM-1 was analyzed using confocal microscopy. Top, Confocal X-Y images of endothelial cell ICAM-1, actin-GFP, two-color overlay, and DIC show clustering of actin and ICAM-1 beneath a PMN that is stably adherent to (but not transmigrating through) the HUVEC monolayer. Bottom panels, Show the side view projections (X-Z) of actin-GFP and ICAM-1 from a series of X-Y images. The horizontal white line in the top panels (X-Y) indicates the position of the X-Z slice. The arrow identifies actin-GFP and ICAM-1 clusters beneath the adherent PMN. Bar, 10 μm. B, Representative X-Y confocal sections of endothelial ICAM-1 (red, left panels) and actin-GFP (green, middle panels) during PMN transmigration. The endothelial cell monolayer was scanned in 0.5-μm steps (apical, middle, and basolateral surfaces). The right panels are merged images of ICAM-1 (red), actin-GFP (green), and DIC (blue), indicating the clustering of ICAM-1 and actin-GFP into ring-like structures around a transmigrating PMN. Data are representative of three independent experiments. Bar, 10 μm.
**Image acquisition and analysis**

Live cell imaging of endothelial ICAM-1 distribution and of leukocyte adhesive interactions with the endothelium was performed using a digital imaging system coupled to a Nikon TE2000 inverted epifluorescence microscope equipped for differential interference contrast (DIC) microscopy (×20 objective, 0.75 NA), as previously described (2). Paired images of DIC and ICAM-1 epifluorescence were acquired during PMN interactions with HUVEC under flow, and the number of adhered and transmigrated leukocytes was determined, as described (3, 15). The percentage of TEM was defined as total transmigrated leukocytes/(adhered PMN + transmigrated PMN) × 100. Clustering of ICAM-1 around PMN was analyzed by overlaying paired ICAM-1 and DIC images using MetaMorph version 4.6c software. ICAM-1 clustering was identified by intense ring-like structures that surrounded stably adherent PMN or PMN in the process of TEM. The intensity of ICAM-1 clustering (Fig. 6C) was calculated by dividing the fluorescence signal surrounding each adherent PMN by the fluorescent signal adjacent to the PMN (baseline) and expressing this value as fold increase.

**Indirect immunofluorescence and confocal microscopy**

Endothelial monolayers were fixed with 10% formalin for 8 min at room temperature. After rinsing three times with DPBS5, where indicated, cells were permeabilized with 0.2% Triton X-100 in PBS for 2 min. Cells were incubated with 1 U/ml Alexa Fluor 543-phalloidin, either alone or (for double staining) combined with 1 μg/ml Alexa Fluor 488-tagged ICAM-1 mAb for 1 h at 4°C. Cells were washed three times with DPBS7 and mounted on glass slides with FluorSave (Calbiochem). Images were analyzed using fluorescence microscopy.

Confocal microscopy was performed using a LSM 510 META laser-scanning confocal head attached to an Axiovert 200 M microscope through a ×63 oil immersion objective (PlanApo, 1.4 NA; Zeiss). The confocal iris was set to provide a 0.5-μm slice thickness, and adherent or transmigrating PMN were scanned along the z-axis at 0.5-μm steps. Images were analyzed in ImageJ version 1.31, and figures were generated in Photoshop version 7.0.

**Data and statistical analysis**

Data are expressed as mean ± SD for three separate experiments (otherwise indicated) and were compared using Student’s t test for paired samples or one-way ANOVA for multiple groups. Data were considered statistically significant if p < 0.05.

**Results**

**Adherent and transmigrating PMN are surrounded by ring-like structures composed of endothelial cell actin and ICAM-1**

Rapid remodeling of the endothelial cell cytoskeleton is critical for leukocyte transmigration (reviewed in Ref. 23). We examined the extent of endothelial cell actin remodeling and ICAM-1 redistribution during PMN adhesion and transmigration in a well-characterized in vitro flow model equipped for live cell imaging (2, 3, 15). HUVEC were transduced with GFP fused to β-actin (actin-GFP) and prelabeled with Alexa 568-conjugated, nonblocking ICAM-1 mAb CL203.4. Expression of actin-GFP (80% infection efficiency) did not alter the levels of endothelial adhesion molecules after TNF treatment or cell-cell junction formation, and did not affect the kinetics or extent of PMN adhesion and transmigration (data not shown). PMN were drawn across the HUVEC monolayer under flow conditions and allowed to interact with HUVEC for 5 min. HUVEC were then fixed, and the tissue was mounted for examination by two-color confocal microscopy. This analysis showed that actin-GFP and ICAM-1 clustered beneath stably adherent PMN (Fig. 1A) and formed ring-like structures that surrounded PMN in the process of transmigration (Fig. 1B). Interestingly, ICAM-1 colocalized with the actin-GFP ring-like structures (Fig. 1B), as assessed by both X-Y sections and the X-Z orthogonal views using confocal microscopy. These studies show that...
endothelial cell ICAM-1 colocalizes with the actin cytoskeleton at sites of PMN adhesion and during PMN transmigration.

**ICAM-1 diffusion on the apical endothelial surface is restricted by interaction of its cytoplasmic domain with the cytoskeleton**

To gain insight into the mechanisms that control the lateral mobility of ICAM-1 and its putative association with the actin cytoskeleton, we used FRAP to measure the mobility of ICAM-1 on the apical surface of HUVEC before and after anti-ICAM-1 Ab cross-linking. The strategy of cross-linking surface ICAM-1 molecules has been used previously to mimic ICAM-1 engagement (5, 24–26). FRAP can be used to determine whether a fluorescently tagged molecule diffuses freely in a cell membrane or whether it interacts with other structural component(s) such as the cytoskeleton. HUVEC cell lines (iHUVEC (17)) expressing similar levels of wild-type ICAM-1GFP or cytoplasmic domain-deleted ICAM-1GFP, termed tailless ICAM-1GFP, were prepared as described (3). In cells subjected to photobleaching under baseline (control) conditions, both wild-type ICAM-1GFP (Fig. 2A) and tailless ICAM-1GFP (data not shown) diffused into the bleached area from the surrounding unbleached area, resulting in recovery of fluorescence (Fig. 2B). The lateral diffusion coefficient of wild-type ICAM-1GFP was calculated to be \(2.9 \times 10^{-10} \text{cm}^2/\text{s}\). The diffusion coefficient of tailless ICAM-1GFP was 3- to 4-fold greater than that of wild-type ICAM-1GFP (Fig. 2C). This difference may be explained by interaction of wild-type ICAM-1GFP with other proteins, such as one or more of the cytoskeletal components cortactin, \(\alpha\)-actinin, ezrin, or moesin (6), through its cytoplasmic domain.

We next assessed the effect of ICAM-1 ligation on its lateral mobility by cross-linking the receptor with an anti-ICAM-1 Ab. Cross-linking of ICAM-1 reduced the lateral diffusion coefficient of wild-type ICAM-1GFP by >50% (Fig. 2, A and C), whereas the mobility of tailless ICAM-1GFP was unaffected by cross-linking (Fig. 2C). We conclude that ligation of ICAM-1 reduces the lateral mobility of this receptor most likely through increased association of its cytoplasmic tail with the cytoskeleton. In this context, the mobility of LFA-1 in resting PBL is constrained by attachment to the cytoskeleton \((D = 3.3 \times 10^{-10} \text{cm}^2/\text{s})\), as measured by FRAP (27). Upon activation by PMA, however, LFA-1 is released from the cytoskeleton and the mobility of LFA-1 increases ~2-fold (27).

**Cortactin contributes to ICAM-1-induced cytoskeletal remodeling**

We next assessed the remodeling of actin-GFP in live HUVEC monolayers in response to ICAM-1 cross-linking. Fluorescence microscopy showed that after cross-linking of ICAM-1 for 10 min, ICAM-1 was distributed as numerous small puncta on the cell surface and actin-GFP formed noticeable fibers across the cell body (Fig. 3A). Quantitation of these experiments revealed that ICAM-1 cross-linking led to a dramatic increase in the percentage of cells containing actin-GFP fibers (no ICAM-1 cross-linking, 12 ± 2%; after ICAM-1 cross-linking, 59 ± 5%). Similar results were obtained in nontransduced HUVEC after ICAM-1 engagement, fixation, and staining with Alexa 545-phallolidin to label F-actin (data not shown). These data are consistent with previous reports using human and rat endothelial cells (5, 24–26). We next confirmed in our model that ICAM-1-triggered cytoskeletal remodeling requires the ICAM-1 cytoplasmic domain. Cross-linking of wild-type ICAM-1GFP caused robust actin cytoskeleton remodeling similar to that seen in primary HUVEC triggered by cross-linking of endogenous ICAM-1, whereas cross-linking of tailless ICAM-1GFP evoked minimal changes in the actin-staining pattern (Fig. 3, B and C).

Because cortactin associates with ICAM-1 and cortactin is a known regulator of the endothelial cell actin cytoskeleton (13, 14), we used siRNA directed against cortactin to determine whether cortactin is involved in ICAM-1-triggered cytoskeletal remodeling. Treatment of HUVEC with cortactin siRNA consistently reduced cortactin levels by 60–70%, as determined by Western blot analysis and by immunofluorescence staining for cortactin (Fig. 4A). We confirmed that cortactin siRNA did not affect the surface expression of ICAM-1, VCAM-1, or E-selectin induced by 4-h treatment with TNF-\(\alpha\) and did not affect the density or location of VE-cadherin (data not shown), as previously reported (15). By
fluorescence imaging, actin-GFP underwent remodeling after ICAM-1 engagement in HUVEC pretreated with scrambled siRNA. In contrast, treatment with cortactin siRNA dramatically reduced actin-GFP fiber formation (Fig. 4B). This result was reproduced in cytokine-activated HUVEC that were fixed and permeabilized, and endogenous F-actin was detected by staining with Alexa 546-conjugated phalloidin, as described in Materials and Methods. The percentage of cells with actin fibers was determined. Analysis of actin fiber formation was measured in at least 100 cells from three separate experiments. */p < 0.01.

Cortactin phosphorylation by Src family kinases is important for ICAM-1-induced cytoskeletal remodeling

Src-induced phosphorylation regulates cortactin function and modulates endothelial cell mobility (13, 14, 28, 29) and leukocyte transmigration (15). We determined whether cortactin phosphorylation by Src is involved in ICAM-1-triggered cytoskeletal remodeling by preincubation of HUVEC with PP2, a well-described inhibitor of Src family kinases (30). PP2 pretreatment abolished tyrosine phosphorylation of cortactin triggered by ICAM-1 cross-linking (data not shown) and largely prevented actin remodeling (Fig. 5A). To corroborate the requirement for cortactin tyrosine phosphorylation, we used a mutant murine cortactin, called cortactin3F-GFP (13), in which tyrosine residues 421, 466, and 482 are mutated to phenylalanine. Murine and human cortactin are highly homologous, and previous studies have reported that the murine wild-type cortactin-GFP is functional in human cells, whereas the cortactin3F-GFP mutant has been shown previously to act in a dominant-negative fashion in endothelial cell ICAM-1 and F-actin clustering during the more relevant endpoint of PMN adhesion and transmigration.

Initial studies used confocal microscopy to quantify the distribution of murine wild-type cortactin-GFP in HUVEC during transmigration of PMN in the in vitro flow model. The data showed that cortactin-GFP forms ring-like structures that surround transmigrating PMN and that these cortactin-GFP rings colocalize with ICAM-1 (Fig. 6A).

Two-color confocal fluorescence microscopy was used to determine the effect of cortactin knockdown on actin remodeling and ICAM-1 clustering at sites of PMN adhesion after 2 min of perfusion in actin-GFP-transduced and ICAM-1-immunolabeled HUVEC pretreated with scrambled or cortactin siRNA (Fig. 6, B and C). Compared with scrambled siRNA, cortactin siRNA dramatically reduced the level of actin clustering in response to PMN adhesion in this time frame (Fig. 6B). This result indicates that cortactin is involved in actin remodeling at sites of PMN adhesion.

Using the same strategy, ICAM-1 clustering was assessed. ICAM-1 formed ring-like clusters at sites of PMN adhesion in HUVEC treated with scrambled siRNA (Fig. 6C), similar to the results presented in Fig. 1B. In contrast, cortactin siRNA caused a reduction in the percentage of PMN that are associated with ICAM-1 ring-like structures (Fig. 6C). Interestingly, the lack of
Endothelial cell ICAM-1 participates in leukocyte adhesion and transmigration. In the current study, we describe a signaling pathway in endothelium that is initiated by engagement of ICAM-1 and that links tyrosine phosphorylation of cortactin to cytoskeletal remodeling and leukocyte adhesion and transmigration. We demonstrate the following: 1) endothelial cell F-actin, cortactin, and ICAM-1 colocalize in ring-like structures (clusters) surrounding actively transmigrating PMN; 2) ICAM-1 lateral mobility in the plasma membrane of endothelial cells is significantly reduced upon ICAM-1 engagement, and is dependent on an intact ICAM-1 cytoplasmic tail; 3) tyrosine phosphorylation of cortactin by Src family kinases is required for optimal ICAM-1-triggered remodeling of the actin cytoskeleton and links ICAM-1 to the newly formed actin cytoskeleton upon ICAM-1 cross-linking; and 4) cortactin mediates cytoskeletal remodeling and ICAM-1 clustering at sites of PMN adhesion, which is important for PMN transmigration through cell-cell junctions. Thus, this study is the first demonstration that endothelial cell cortactin serves as a key integrator for ICAM-1 outside-in signaling and the first characterization of ICAM-1 clustering caused by cortactin siRNA was accompanied by impaired actin fiber clustering at the site of PMN adhesion (Fig. 6, B and C), suggesting a correlation between actin and ICAM-1 clustering. The degree of ICAM-1 fluorescence intensity in clusters relative to the baseline fluorescence in which no PMN had bound was quantified. ICAM-1 showed a significant increase (3-fold; \( p < 0.05; n = 100 \) cells from three separate experiments) around PMN adherent to HUVEC treated with scrambled siRNA as compared with HUVEC treated with cortactin siRNA (Fig. 6D, right panel). Cortactin siRNA-impaired ICAM-1 clustering was rescued by expressing wild-type cortactin-GFP in HUVEC treated with cortactin siRNA (Fig. 6E). Together with the results presented in Figs. 4 and 5, these observations suggest that cortactin mediates cytoskeletal remodeling, which in turn regulates ICAM-1 clustering around adherent PMN.

**Cortactin-dependent ICAM-1 clustering around adherent PMN is required for transmigration**

We next asked whether ICAM-1 clustering in HUVEC is necessary for PMN transmigration. For this analysis, PMN interactions with endothelial cells and ICAM-1 clustering were monitored simultaneously by live cell DIC microscopy and single color fluorescence microscopy. In preliminary experiments, we found that HUVEC immunolabeled with Alexa 568-tagged ICAM-1 mAb were sometimes damaged (i.e., endothelial cell retraction was observed) by prolonged illumination for fluorescence imaging. We therefore modified the imaging protocol to obtain paired images of ICAM-1 fluorescence and DIC at 15-s intervals for the first 2 min of PMN perfusion, and thereafter, we obtained only DIC (nonfluorescent light) images for an additional 8 min. This scheme allowed us to correlate ICAM-1 clustering around adherent PMN with the ability of these PMN to transmigrate. Occasional fluorescence imaging was used to track migrating PMN, and hence monitor both ICAM-1 clustering and PMN transmigration. Overlaying of the paired DIC and ICAM-1 fluorescence images clearly demonstrated ICAM-1 clustering around many PMN adherent to HUVEC pre-treated with scrambled siRNA (Fig. 7A, PMN identified as 1–4). These PMN with ICAM-1 clustering did subsequently transmigrate. In contrast, significantly fewer PMN were associated with ICAM-1 clusters in HUVEC treated with cortactin siRNA (Fig. 7A, PMN identified as 1–2). We noted that PMN with robust ICAM-1 clustering subsequently transmigrated, whereas PMN with minimal or undetectable ICAM-1 ring-like clusters did not transmigrate. Analysis of three separate experiments confirmed this outcome and showed that cortactin siRNA significantly reduced PMN transmigration (Fig. 7B; 52% inhibition of TEM), but did not alter PMN adhesion under defined shear stress. Analysis of four independent experiments showed a high degree of correlation between ICAM-1 clustering and PMN transmigration (Fig. 7B), strongly supporting an important role for cortactin-dependent ICAM-1 clustering during transmigration.

**Discussion**

Endothelial cell ICAM-1 participates in leukocyte adhesion and transmigration. We demonstrate the following: 1) endothelial cell F-actin, cortactin, and ICAM-1 colocalize in ring-like structures (clusters) surrounding actively transmigrating PMN; 2) ICAM-1 lateral mobility in the plasma membrane of endothelial cells is significantly reduced upon ICAM-1 engagement, and is dependent on an intact ICAM-1 cytoplasmic tail; 3) tyrosine phosphorylation of cortactin by Src family kinases is required for optimal ICAM-1-triggered remodeling of the actin cytoskeleton and links ICAM-1 to the newly formed actin cytoskeleton upon ICAM-1 cross-linking; and 4) cortactin mediates cytoskeletal remodeling and ICAM-1 clustering at sites of PMN adhesion, which is important for PMN transmigration through cell-cell junctions. Thus, this study is the first demonstration that endothelial cell cortactin serves as a key integrator for ICAM-1 outside-in signaling and the first characterization of...
FIGURE 6. Cortactin siRNA impairs clustering of actin and ICAM-1 around adherent PMN. A, Confluent HUVEC monolayers expressing wild-type cortactin-GFP were transfected with scrambled or cortactin siRNA, were activated for 4 h with TNF-α, and PMN were drawn through the chamber for 5 min before the experiment was terminated by fixation (10% formalin-buffered saline). Colocalization of cortactin-GFP with endothelial ICAM-1 is demonstrated by confocal microscopy. Confocal X-Y and X-Z views reveal that ICAM-1 and cortactin colocalize in a ring-like structure at sites of PMN transmigration. Bar, 10 μm. B and C. HUVEC monolayers transfected with actin-GFP were transfected with scrambled or cortactin siRNA, as described in Materials and Methods. These HUVEC were activated for 4 h with TNF-α, and ICAM-1 was labeled as above in A and then inserted into the flow model. PMN were drawn across the monolayers for 2 min, and monolayers were fixed with 10% buffered formalin. The distribution of actin-GFP (B) and ICAM-1 (C) was analyzed by two-color confocal microscopy, and clustering of ICAM-1 and actin-GFP was quantified for >100 PMN group from three separate experiments. *, p < 0.01. D, Quantitation of ICAM-1 clustering around adherent PMN on HUVEC monolayers treated with scrambled or cortactin siRNA. Left panels, DIC and accompanying ICAM-1 image demonstrating enrichment of ICAM-1 in ring-like structure surrounding an adherent PMN. The solid white line is positioned across the center of the adherent PMN. The intensity profile of the line shows peaks of high intensity corresponding to ICAM-1 concentration at the site of PMN adherent to scrambled siRNA-treated HUVEC as compared with low-intensity peaks in PMN bound to cortactin siRNA-treated HUVEC, as indicated. Right graph, To compare ICAM-1 fluorescence in cortactin vs scrambled siRNA-treated HUVEC, PMN-associated ICAM-1 fluorescence from 100 adherent PMN (three separate experiments) was quantified and compared with the background fluorescence in the same size region of interest adjacent to the adherent PMN in which no PMN was bound. Bar, 10 μm. *, p < 0.01. E, HUVEC were transduced with wild-type murine cortactin-GFP or GFP (as control) and treated with scrambled or cortactin siRNA, as indicated. Clustering of ICAM-1 was determined for at least 100 PMN. Data were from three separate experiments. *, p < 0.01, vs treatment with scrambled siRNA. NS, vs treatment with scrambled siRNA.

the role of cortactin in cytoskeletal remodeling, leukocyte-endothelial docking structure formation, and leukocyte transmigration under shear flow conditions. Previous studies have shown that endothelial cell F-actin colocalizes with ICAM-1 and VCAM-1, in concert with the actin-binding proteins moesin and ezrin, α-actinin, talin, and vinculin, to form docking structures (8, 31) that surround adherent leukocytes and cup-like projections (4, 9) that surround transmigrating leukocytes. Moesin and ezrin and the p160ROCK signaling pathway have been implicated in the generation and maintenance of lymphoblast-endothelial cell docking structures (8), although their role in transmigration has not been determined. ICAM-1 is known to associate with a variety of actin-binding proteins, including α-actinin, phospho-ezrin, phospho-moesin, and cortactin. Our laboratory has recently shown that Src kinase phosphorylation of endothelial cortactin is an important mechanism that regulates PMN transmigration at cell-cell junctions (paracellular transmigration) under flow conditions (15). Live cell imaging demonstrates that cortactin-GFP forms small clusters around adherent and transmigrating PMN and redistributes to sites of endothelial cell-cell junctions that reseal after completion of transmigration (15). These findings, taken together, led us to test whether the Src kinase-cortactin pathway is also involved in ICAM-1 clustering and actin cytoskeleton remodeling during leukocyte TEM. In this study, we show that siRNA knockdown of cortactin or inhibition of cortactin tyrosine phosphorylation dramatically impairs cytoskeleton remodeling induced in endothelial cells by ICAM-1 cross-linking (Figs. 4 and 5). Although cortactin siRNA has no effect on PMN adhesion at the low physiological fluid shear stress used in this study (Fig. 7A) and previously (15), it dramatically reduces ICAM-1 clustering around adherent and transmigrating PMN and, thus, reduces PMN transmigration (Figs. 6 and 7). These data support our hypothesis that cortactin can act as an integrator of ICAM-1 outside-in signaling and as a downstream effector for...
ICAM-1 clustering and cytoskeletal remodeling, both of which are highly correlated with PMN TEM.

The endothelial cell cytoskeleton plays an important role in the process of leukocyte transmigration (reviewed in Ref. 23). In this study, we take advantage of live cell imaging of H9252-actin GFP fusion protein and confocal microscopy analysis to explore the role of cortactin in the temporal and spatial remodeling of endothelial F-actin and ICAM-1 observed during ICAM-1 cross-linking and during PMN adhesion and transmigration. Notably, cross-linking of ICAM-1 triggers robust actin remodeling both at cell-cell junctions and as stress fibers in the HUVEC cell body, and knockdown of cortactin by siRNA dramatically inhibited these changes. The mechanism(s) that mediates stress fiber formation is thought to involve a small GTPase and formins and incorporation of myosin, but the assembly of these components into actin stress fibers remains incompletely understood (32–34). The current data strongly implicate a contribution of cortactin to ICAM-1 cross-linking-dependent stress fiber formation in vascular endothelium.

As discussed earlier, whereas ICAM-1 cross-linking is a convenient approach and may achieve maximum ICAM-1 occupancy, one caveat is that Ab cross-linking does not completely recapitulate the transient and focal engagement of ICAM-1 during neutrophil adhesion and transmigration. Hence, our in vitro flow model was used to evaluate the role of cortactin in actin cytoskeleton remodeling during PMN transmigration. Interestingly, actin-GFP forms transient, robust ring-like structures that colocalize with similar ring-like structures of both ICAM-1 and cortactin surrounding adherent and transmigrating PMN (Figs. 1A and 6A). Cortactin siRNA knockdown significantly disrupts clustering of both endothelial cell F-actin and ICAM-1 around adherent PMN (Figs. 6 and 7). We speculate that these ring-like F-actin structures arise from an interaction among cortactin and the Arp2/3 complex and additional regulatory molecules, such as members of the Wiskott-Aldrich syndrome protein family (12, 35). The current data strongly implicate a contribution of cortactin to ICAM-1 cross-linking-dependent stress fiber formation in vascular endothelium.

FIGURE 7. PMN transmigration is significantly impaired by cortactin knockdown. HUVEC were transfected with cortactin or scrambled siRNA and activated with TNF-α for 4 h. ICAM-1 was prelabeled on these HUVEC, as described above. HUVEC were allowed to interact with PMN under flow. Images of ICAM-1 fluorescence and corresponding DIC images were taken for the first 2 min of flow at 15-s intervals, followed by DIC imaging only for an additional 8 min. A, Adherent PMN (2 min, top row, DIC) with or without ICAM-1 clustering (middle row, red) were identified by overlay of DIC and ICAM-1 fluorescent images (bottom row). Whether these PMN transmigrated was tracked by DIC imaging during 10 min of flow. PMN with ICAM-1 clustering were indicated by arrows. Adherent PMN labeled with numbers at 2 min of flow are identical with those with the same number at 10 min. Bar, 20 μm. B, The percentage of PMN associated with ICAM-1 clustering during adhesion (determined at 2 min of flow) and PMN transmigration after 10 min was determined in HUVEC treated with cortactin or scrambled siRNA, as described in A. r² = 0.98. For each group, at least 100 cells from three separate experiments were analyzed.

FIGURE 8. A model of the ICAM-1-Src-cortactin signaling pathway that regulates cytoskeletal remodeling, ICAM-1 clustering, and PMN transmigration. A, ICAM-1 is engaged by its ligands on PMN. B, Engagement of ICAM-1 results in recruitment of Src family kinases and cortactin to the ICAM-1 cytoplasmic domain, and cortactin is phosphorylated by Src. C, Src-induced cortactin phosphorylation leads to temporal and spatial actin remodeling. D, The newly formed F-actin stabilizes clustered ICAM-1, which leads to an intense colocalization of ICAM-1 with its ligands and initiation of amplified signaling between the leukocyte and the endothelium. E, In endothelium, strengthened interaction between ICAM-1 and its ligands causes cytoskeletal contraction, which facilitates PMN transmigration.
a branched actin network in the leading front of motile cells (reviewed in Ref. 32). These data, taken together, provide definitive evidence that cortactin is intimately involved in the generation and the maintenance of endothelial-leukocyte adhesion structures described previously (docking structures (8, 23)). Our current data also argue for a more complex mechanism of regulation of docking structures than previous models that involved only ezrin, moesin, and p160ROCK (31). The interplay and regulation of the src-cortactin pathway with these other actin-linking proteins and their signaling pathways during leukocyte adhesion and transmigration deserve further study.

Our data point to a causal relationship between cortactin-mediated ICAM-1 clustering and PMN transmigration (Figs. 6 and 7). We show that ICAM-1 clustering around adherent and transmigrating PMN is regulated by cortactin-mediated cytoskeletal remodeling because cortactin siRNA knockdown by siRNA impairs both actin and ICAM-1 ring-like structure formation and significantly reduces PMN transmigration. Because the level of cortactin knockdown achieved was only ∼70%, the observed decreases in cortactin-dependent clustering of F-actin and ICAM-1 and PMN transmigration may be underestimates. Whether endothelial ICAM-1-enriched docking structures progress to form a transmigration cup has yet to be elucidated; nonetheless, the current findings support a dominant role for cortactin in each of these structures. We speculate that cortactin-mediated clustering of ICAM-1 acts as a potent stimulus for leukocyte LFA-1 and facilitates conformational changes that lead to increases in the avidity and/or affinity of LFA-1 for ICAM-1. This, in turn, could amplify signaling in both the leukocyte and endothelial cell, triggering the leukocyte to extend lamellipodia between endothelial cell–cell junctions to initiate formation of gaps or pores in endothelial cell junctions for leukocyte TEM (TEM tunnels) (Fig. 8). We propose a model of the signaling pathway from ICAM-1 engagement to the cytoskeleton through Src family kinases and cortactin, leading to leukocyte TEM (Fig. 8).

The current study suggests a critical role for cortactin in paracellular transmigration. Previous reports have demonstrated that blood leukocytes can transmigrate at cell–cell borders (paracellular transmigration) and through the endothelial cell (transcellular migration), as assessed in vivo and in vitro models of inflammation (3–5, 36, 37). Recent models of transcellular transmigration identify ICAM-1 clustering during transmigration as a critical event during transmigration (3–5). Interestingly, endothelial cell caveolin-1 and vimentin, an intermediate filament protein, have been implicated in ICAM-1 clustering and leukocyte transcellular transmigration (5, 36). Knockdown of caveolin-1 by siRNA partially inhibited in vitro derived T lymphoblast transcellular transmigration. Millán et al. (5) proposed that transcellular TEM of T lymphoblast occurs at sites in which ICAM-1 is associated with F-actin and caveola-rich regions, which give rise to a transcellular pore. In contrast, in the vimentin knockout mouse (36), the loss of endothelial vimentin causes decreased barrier function and aberrant expression and distribution of ICAM-1 and VCAM-1 and a similar disruption of β1 integrins in T cells. Thus, future studies are needed to determine the contributions of F-actin, cortactin, vimentin, and caveolin-1 to the paracellular and transcellular transmigration pathways, and to identify the signal cascade that regulates these two modes of transmigration.

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Disclosures
The authors have no financial conflict of interest.

References


