

Endothelial-dependent Mechanisms Regulate Leukocyte Transmigration: A Process Involving the Proteasome and Disruption of the Vascular Endothelial-Cadherin Complex at Endothelial Cell-to-Cell Junctions

By Jennifer R. Allport,* Han Ding,* Tucker Collins,*
Mary E. Gerritsen[†] and Francis W. Luscinskas*

From *The Vascular Research Division, Departments of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115; and [†]Bayer Corporation, West Haven, Connecticut 06516

Summary

Although several adhesion molecules expressed on leukocytes (β 1 and β 2 integrins, platelet endothelial cell adhesion molecule 1 [PECAM-1], and CD47) and on endothelium (intercellular adhesion molecule 1, PECAM-1) have been implicated in leukocyte transendothelial migration, less is known about the role of endothelial lateral junctions during this process. We have shown previously (Read, M.A., A.S. Neish, F.W. Luscinskas, V.J. Palambella, T. Maniatis, and T. Collins. 1995. *Immunity*. 2:493–506) that inhibitors of the proteasome reduce lymphocyte and neutrophil adhesion and transmigration across TNF- α -activated human umbilical vein endothelial cell (EC) monolayers in an in vitro flow model. The current study examined EC lateral junction proteins, principally the vascular endothelial (VE)-cadherin complex and the effects of proteasome inhibitors (MG132 and lactacystin) on lateral junctions during leukocyte adhesion, to gain a better understanding of the role of EC junctions in leukocyte transmigration. Both biochemical and indirect immunofluorescence analyses of the adherens junction zone of EC monolayers revealed that neutrophil adhesion, not transmigration, induced disruption of the VE-cadherin complex and loss of its lateral junction localization. In contrast, PECAM-1, which is located at lateral junctions and is implicated in neutrophil transmigration, was not altered. These findings identify new and interrelated endothelial-dependent mechanisms for leukocyte transmigration that involve alterations in lateral junction structure and a proteasome-dependent event(s).

Localized leukocyte accumulation is the cellular hallmark of inflammation. Although this has been recognized for more than a century, it is only in the past decade that the role of the endothelium has been appreciated. The notion that the vascular endothelium actively participates in leukocyte recruitment initially gained support from in vitro studies demonstrating that treatment of cultured endothelium with inflammatory cytokines TNF- α or IL-1, and certain Gram-negative bacterial endotoxin could “activate” the endothelium to become adhesive for blood leukocytes and cell lines (1–3). Subsequently, the work of many investigators has identified and molecularly cloned several such endothelial cell (EC)¹ adhesion molecules and

their cognate ligands on leukocytes, which support leukocyte adhesion to endothelium.

Recent reports have shown that the proteasome pathway is involved in activation of NF- κ B, which is a transcription factor necessary for activation of EC gene transcription of E-selectin (CD62E), intercellular adhesion molecule 1 (ICAM-1) (CD54), and vascular cell adhesion molecule 1 (VCAM-1) (CD106) (4). Small peptide aldehyde inhibitors (MG132, MG115) of the proteasome can dramatically reduce TNF- α -induced expression of E-selectin, VCAM-1, and ICAM-1 in human umbilical vein ECs (4). Functionally, neutrophil adhesion was reduced by 50%, and transmigration was reduced by >60%. Live-time video microscopy showed that many adherent neutrophils had flattened and extended pseudopods into the EC junctions, but were

¹Abbreviations used in this paper: ALLM, *N*-acetyl-leucyl-leucyl-methional; DPBS, Dulbecco's phosphate-buffered saline; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; HSA, human serum albumin; ICAM, intercellular adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule 1; TBS, Tris-buffered saline; VCAM 1, vascular cell adhesion molecule 1.

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unable to transmigrate. This result raises the possibility that the proteasome regulates an essential endothelial-dependent component(s) during transendothelial passage.

In light of our previous observations that proteasome inhibitors prevent firmly adherent neutrophils from penetrating between endothelial cell–cell lateral junctions, we reasoned that the function of endothelial cell-to-cell lateral junctions may be critical during the process of leukocyte transmigration. The molecular structure and organization of endothelial cell–cell lateral junctions has been reviewed (5–7). We focused our attention on the adherens type junctions which appear to serve as a focal point for the connections between the EC plasma membrane and its underlying actin-cytoskeleton complex. The adherens type junctions contain cadherins (for review see reference 7), a family of single-span transmembrane glycoproteins which directly associate with structural components of the cytoskeleton and mediate Ca^{2+} -dependent cell–cell adhesion in a homotypic fashion. Cadherin-5, also termed vascular endothelial (VE)-cadherin, is specific to vascular endothelium and localizes exclusively to lateral junctions of intact, confluent endothelium (8, 9). A recent study (8) has revealed that VE-cadherin associates with the cytosolic proteins α - and β -catenins to form a complex and organize at nascent endothelial cell-to-cell contacts. Plakoglobin (also named γ -catenin) associated with VE-cadherin and α - and β -catenins at cell-to-cell contacts, through an undefined mechanism(s), only as EC approached confluence. p120 (p120^{cas}), initially identified as one of several substrates of the tyrosine kinase pp60^{src} (10, 11), and a closely associated molecule termed p100 also have been reported to associate with α - and β -catenins and VE-cadherin in umbilical vein endothelium (12). That the VE-cadherin complex is dynamic and involved in regulating cell-to-cell contact is suggested by wounding (8) or Ca^{2+} depletion (5) experiments where VE-cadherin and plakoglobin rapidly and reversibly retract from the endothelial lateral junctions.

To better understand the molecular basis of inhibition of neutrophil transmigration by proteasome inhibitors and the potential role of the components of the EC adherens junctions, we have evaluated the effects of two structurally different proteasome inhibitors, MG132 and lactacystin (13), on the association of VE-cadherin with α -, β -, and γ -catenin (plakoglobin), and p120/p100 in 6-h TNF- α -activated EC using both indirect immunofluorescence microscopy and immunoprecipitation followed by immunoblotting. Second, we have coinubated human neutrophils with control or proteasome inhibitor-treated TNF- α -activated endothelium, and evaluated the staining patterns and biochemical association of members of the VE-cadherin complex, and as a control, platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31), which has been shown previously to colocalize to EC lateral junctions (14) and has been implicated in leukocyte transmigration in *in vivo* and *in vitro* studies (15). The results of these experiments suggest that novel endothelial-dependent mechanisms regulate neutrophil transmigration which involves structural alterations of the VE-cadherin complex, as well as a proteasomal-dependent step(s).

Materials and Methods

Materials

Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+} and Mg^{2+} , DPBS, M199, DMEM, RPMI-1640 with 25 mM Hepes, and 1 M solution of Hepes were purchased from BioWhittaker Bioproducts (Walkersville, MD). Human rTNF- α (produced in *Escherichia coli*) was the gift of Dr. Baker (Genentech Inc., South San Francisco, CA), and a concentration of 25 ng/ml gave maximal response and contained <10 pg/ml of endotoxin as reported previously (16). The proteasome inhibitors, MG132 (carbobenzoxyl-leuciny-leuciny-leucinal-H) and lactacystin were the gift of Dr. J. Adams (ProScript, Inc., Cambridge, MA). Each inhibitor was dissolved in DMSO at 40 mM and stored at -80°C . For use in experiments, aliquots of inhibitors were thawed at 37°C and diluted directly into appropriate culture media, or as otherwise noted in the text. Calpain inhibitor II (also abbreviated as ALLM [*N*-acetyl-leuciny-leuciny-methional]) and aprotinin were purchased from Calbiochem Corp. (La Jolla, CA). EDTA, Protein-G Sepharose, PMSF, leupeptin, DMSO, BSA, and Hepes were purchased from Sigma Chemical Co. (St. Louis, MO). Human serum albumin (HSA) was obtained from Baxter Healthcare Corp. (Glendale, CA).

mAbs

The following murine mAbs have been reported previously: anti-E-selectin (H4/18 or 7A9, each an IgG₁; reference 16), anti-ICAM-1 (Hu5/3, IgG₁; reference 17), anti-VCAM-1 mAb E1/6 (IgG₁; reference 18), anti-PECAM-1 (obtained from Iowa Hybridoma Bank, Iowa City, IA, or from Immunotech, Inc., Westbrook, ME), and anti-HLA-A,B (W6/32, IgG_{2a}) mAb (19). These mAb were used as hybridoma culture supernatant fluid for surface immunofluorescence assays and as pure IgG for immunofluorescence microscopy, immunoprecipitations, and Western blotting. Murine mAb directed to VE-cadherin (clone TEA1/31, IgG₁) was purchased from Immunotech Inc. or from BIODESIGN Intl. (Kennebunk, ME). Murine mAb directed to plakoglobin (clone PG5.1, IgG_{2b}) was purchased from BIODESIGN Intl. Murine mAb to α - and β -catenins and p120 (clone 5, 14, and 98, respectively; all IgG₁) were purchased from Transduction Labs. (Lexington, KY). A second mAb to α -catenin (Zymed, S. San Francisco, CA) was used for immunofluorescence studies.

Cell Culture

ECs were isolated from two to five umbilical cord veins, pooled, and established as detailed (19). Primary human umbilical vein endothelial cell (HUVEC) cultures were serially passaged (1:3 split ratio) and maintained in M199 containing 10% FCS, EC growth factor, porcine intestinal heparin, and antibiotics.

Experimental Protocols. For immunofluorescence staining assays, ECs (passage 1–2) were plated at confluent density on 4-well chamber glass slides (Lab-Tek; Nunc, Inc., Naperville, IL) until 2 d after confluent. For flow assays, ECs were plated at confluence on human fibronectin ($2\ \mu\text{g}/\text{cm}^2$)-coated 25-mm glass coverslips (20), and used 3 d later. For quantitative surface immunofluorescence assays, ECs were plated on fibronectin-coated microtiter plates (C96; Costar, Cambridge, MA), and used 2–3 d after confluence. For immunoprecipitation assays, EC were plated on 0.1% gelatin-coated 100-mm diameter plastic petri dishes (Costar), and used 3 d after attaining confluence. EC monolayers were not manipulated or fed for 48 h before use.

Table 1. Effects of Proteasome Inhibitor Lactacystin on EC Expression of Adhesion Molecules.

Treatment	E-selectin	ICAM-1	VCAM-1	p96
None	13 ± 22	29 ± 3	2 ± 3	500 ± 10
1 h carrier; TNF- α 4 h	272 ± 29 [†]	239 ± 3 [†]	253 ± 8 [†]	488 ± 28
1 h Lac; TNF- α 4 h	72 ± 7 [§]	72 ± 3 [§]	72 ± 8 [§]	432 ± 42
1 h ALLM; TNF- α 4 h	232 ± 45 [†]	220 ± 3	247 ± 18	428 ± 18
TNF- α 4 h; carrier 2 h	235 ± 73	334 ± 20	352 ± 9	444 ± 42
TNF- α 4 h; Lac 2 h	237 ± 19	350 ± 4	334 ± 22	498 ± 45
TNF- α 4 h; ALLM 2 h	245 ± 20	351 ± 17	343 ± 8	465 ± 45

*Surface expression of adhesion molecules was determined using a two-step indirect surface immunofluorescence assay (19) and the mAb listed in Materials and Methods. Aliquots of lactacystin (*Lac*, 10 mM) and calpain inhibitor II (*ALLM*, 40 mM) were thawed and immediately diluted into culture media to final concentration of 20 μ M. Carrier control was 0.02% DMSO. The results are the average of triplicate wells and are representative of two separate experiments. Background nonbinding control mAb K16/16 was 6 ± 4.

[†]Value is significantly higher ($P < 0.05$) than media control of unactivated EC monolayers

[§]Value is significantly lower than carrier TNF- α -activated EC monolayers ($P < 0.01$).

Leukocyte Isolation

Human neutrophils were purified from whole blood of volunteer donors as previously detailed (19). Isolated neutrophils (94% pure) were resuspended in cold DPBS containing 0.75 mM Ca²⁺, 0.75 mM Mg²⁺, and 0.2% HSA (assay buffer).

Endothelial–Leukocyte Interactions in a Parallel-plate Flow Chamber

The parallel-plate flow chamber used in this study has been described in detail (20–22). A wall shear stress of 1.8 dynes/cm² was achieved with a flow rate of 0.85 ml/min (20). EC monolayers were assayed for leukocyte adhesion and transmigration under flow conditions as before (16, 20).

Immunofluorescence Assays

Quantitative surface immunofluorescence assays for adhesion molecule expression on EC were performed in triplicate wells as reported previously (17) using appropriate primary mAb–detected FITC-conjugated goat anti–mouse (F(ab')₂, 1:50 dilution; Caltag Labs., S. San Francisco, CA).

Indirect Immunofluorescence Staining. Indirect immunofluorescence staining of endothelial surface molecules was performed using the protocol of Gerritsen et al. (23). In brief, confluent EC in 4-well chambers were activated as detailed above and then washed with assay buffer. Neutrophils (0.3 × 10⁶) or assay buffer were added to wells and incubated at 37°C for 10 min. Wells were washed twice with DPBS, and then fixed with methanol (–20°C) on ice for 5 min, followed by three washes. Immunofluorescence staining was performed as detailed (23). Fields of FITC fluorescence–stained EC were visualized on a fluorescence microscope (Microfot FXA; Nikon, Inc., Melville, NY) equipped with a ×20 objective and the images were captured using a cooled charged-coupled device video camera. Exposure times were matched in each instance (typically 1–3 s, final magnification of all images was 320, except where noted).

Immunoprecipitation of Endothelial Proteins and Immunoblotting

Immunoprecipitation of Junctional Proteins. VE–cadherin complex proteins were immunoprecipitated using a modification of

the method of Lampugnani et al. (8). In brief, EC were incubated with inhibitors, ALLM, or carrier (0.02% DMSO) and/or TNF- α , and then washed three times with assay buffer. EC were incubated at 37°C for 10 min under static conditions with either 10⁷ human neutrophils (5 ml, ratio of neutrophils to EC was 2.5:1) or assay buffer alone. Nonadherent neutrophils were removed by washing (2 times) the monolayers with DPBS alone. Plates were placed on ice and the Triton X-100 soluble fraction extracted for 30 min in lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM PMSF, 40 U/ml aprotinin, 15 μ g/ml leupeptin, 0.36 mM 1,10-phenanthroline, 2 mM CaCl₂ [Tris-buffered saline [TBS] and protease inhibitors), 1% NP-40, and 1% Triton X-100), mixing every few minutes. For total lysates, monolayers were lysed in lysis buffer containing 0.5% SDS. The supernatant was collected, microcentrifuged at 14,000 g for 5 min, and stored at –80°C. The lysed monolayers were washed 3 times with TBS and protease inhibitors, and the Triton X-100 insoluble fraction was extracted as detailed (8) and stored at –80°C.

For immunoprecipitation, protein G–Sepharose was washed with TBS, and a 60- μ l suspension (1:1 suspension with TBS) was coupled to mAb by mixing for 1 h at ambient temperature with 8–10 μ g of one of several mAb in a final volume of 500 μ l. The above concentrations of mAbs were optimal for detection in our system. The IgG protein G–Sepharose was microcentrifuged and washed (3 times) with TBS containing 1 mM PMSF, 40 U/ml aprotinin, 1 mM CaCl₂, and 2% (vol/vol) Triton X-100 (buffer 1). The cell lysates (representing 0.5–1 × 10⁶ EC) were precleared by incubating with protein G–Sepharose, pelleted, and the supernatants were incubated with the IgG protein G–Sepharose for 1 h at RT. The immunoprecipitates were then washed (3 times) with TBS containing 1 mM PMSF, 40 U/ml aprotinin, 0.05% (vol/vol) NP-40, and 2 mM CaCl₂, and boiled for 5 min in 2× sample loading buffer. The entire supernatant of samples was resolved by SDS-PAGE (24) on a 7.5% (wt/vol) polyacrylamide gel and transferred to nitrocellulose membrane (Hybond; Amersham Corp., Arlington Heights, IL).

Immunoblot Analysis. Immunoblots were performed using a modification of the method of Donnelly et al. (25). The membrane was blocked for 1 h with 5% dried milk protein in PBS (blocking buffer) and then incubated for 2 h with primary mAb (either 5 μ g/ml anti–VE–cadherin, 1 μ g/ml anti– α -catenin, anti– β -cate-

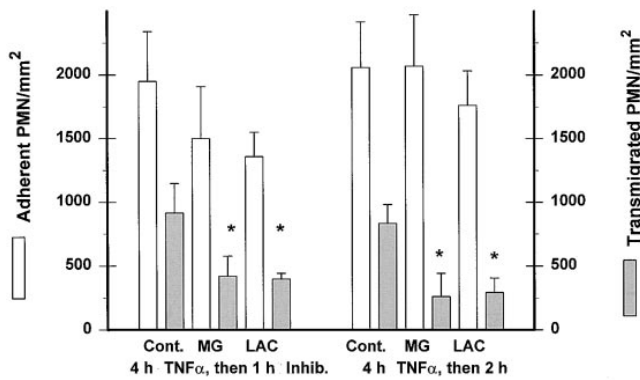


Figure 1. Proteasome inhibitors MG132 and lactacystin prevent neutrophil migration under flow at 1.8 dynes/cm². Confluent EC monolayers were incubated with TNF- α for 4 h before addition of proteasome inhibitors (MG, 20 μ M MG132; LAC, 20 μ M lactacystin) or DMSO carrier (Cont.) for 1 (left) or 2 h (right), and neutrophil adhesion and transmigration were assessed as detailed in Materials and Methods. * $P < 0.05$.

nin or anti-p120, or 5 μ g/ml anti-plakoglobin or anti-PECAM-1 diluted in block). The membrane was washed six times at 5 min intervals with PBS containing 0.05% (vol/vol) Tween 20 and 0.05% (wt/vol) BSA Tween 20, and then the primary mAbs were detected with anti-mouse IgG conjugated to horseradish peroxidase (Sigma Chemical Co., 1/10,000 dilution, 1 h at room temperature). The immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Corp.).

Statistics

Adhesion data was collected by analyses of variance and Student's two sample t test was used to calculate statistical significance (Minitab Software, State College, PA).

Results

Lactacystin Inhibits Neutrophil Transendothelial Migration.

We have found previously that pretreatment of endothelial monolayers with the aldehyde peptide 20S proteasome inhibitor MG132 before TNF- α activation of EC dramatically reduces the induction of E-selectin, ICAM-1, and VCAM-1 gene transcription (4). Functionally, this results in reduced neutrophil and lymphocyte adhesion and inhibition of transendothelial migration under flow conditions. Lactacystin (13) is a *Streptomyces* metabolite that potently inhibits the 20S proteasome, and is structurally distinct from MG132 (4). Pretreatment of EC with lactacystin (20 μ M) before TNF- α also significantly inhibited surface expression of adhesion molecules (Table 1), reduced adhesion by 88%, and essentially ablated neutrophil transmigration (>95% inhibition).

To distinguish between the effects of the proteasome inhibitors on expression of endothelial leukocyte adhesion molecules and the process of neutrophil transmigration, MG132, lactacystin, or carrier control were added to 4 h TNF- α -activated EC, and the monolayers further incubated. Under such conditions, both inhibitors have no effect on neutrophil adhesion (Fig. 1) or expression of endo-

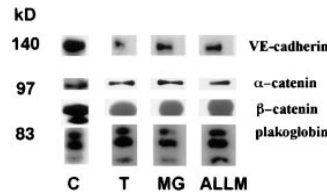


Figure 2. The endothelial VE-cadherin complex is not altered by incubation with proteasomal inhibitors with or without TNF- α treatment. Confluent EC monolayers were treated with inhibitors before or after addition of TNF- α , washed twice, and extracted in lysis buffer 1 (Materials and Methods). The VE-cadherin complex was immunoprecipitated using anti-VE-cadherin mAb, separated by SDS-PAGE, and subjected to immunoblotting using mAbs that recognize α - and β -catenins, plakoglobin, and p120. The results with anti-p120 mAb were variable and p120 was not always detected, even though the other components shown were consistent and reproducible. The x-axis labels are: C, 0.02% DMSO; T, 25 ng/ml of TNF- α for 4 h; MG, MG132 (20 μ M) for 4 h; ALLM, ALLM (20 μ M) treatment for 4 h.

thelial adhesion molecules (Table 1 and data not shown), but reduce neutrophil transmigration by >50% (Fig. 1). The effect on transmigration was observed by 60 min, and by 120 min the level of blockade was >70% for MG132 and >60% for lactacystin. A direct effect of the inhibitors on neutrophils is not likely because a 5-min pretreatment of neutrophils with 5 μ M MG132 before perfusion did not alter neutrophil adhesion or transmigration (vehicle, 51.5 \pm 5.9% transmigrated versus MG132 treated, 51.5 \pm 6.3%; $n = 3$ experiments). We conclude that MG132 and lactacystin, two structurally distinct inhibitors of the proteasome, act on the endothelium to block transmigration, separately from their effect on adhesion molecule expression.

The Endothelial VE-Cadherin Complex Is Not Altered by Treatment with TNF- α , Proteasomal Inhibitors, or Their Combination. Inhibition of neutrophil penetration suggests that the function of lateral junctions is an important endothelial-dependent component(s) for transendothelial passage of the leukocyte. To examine the effects of TNF- α , proteasomal inhibitors or both on the VE-cadherin complex, confluent EC monolayers were incubated with or without inhibitors and then with or without TNF- α for a total of 6 h. The effect of these treatments on the VE-cadherin complex was determined in Triton X-100-soluble lysates by immunoprecipitation with anti-VE-cadherin mAb and immunoblot analysis using specific mAb, and by a second independent analysis using immunofluorescence photomicroscopy. VE-cadherin, α - and β -catenins, and plakoglobin were clearly identified (Fig. 2, lane C), and their migration in SDS-PAGE under reduced conditions was consistent with previous reports (8, 12). Similarly, immunoprecipitation with anti-VE-cadherin mAb, and subsequent immunoblotting analysis with an anti-p120 mAb, detected a single band migrating at 120 kD (data not shown), but the results were not consistently found in every EC culture examined. However, analysis of EC lysates with anti-p120 mAb followed by blotting with anti-p120 consistently revealed a band at 120 kD and a second band at 100 kD, consistent with a recent analysis in endothelium derived from brain (12). The endothelial VE-cadherin complex was not altered by 6 h of incubation with TNF- α , MG132, ALLM, lactacystin, or carrier control (Fig. 2, compare lane C with lanes T, MG,

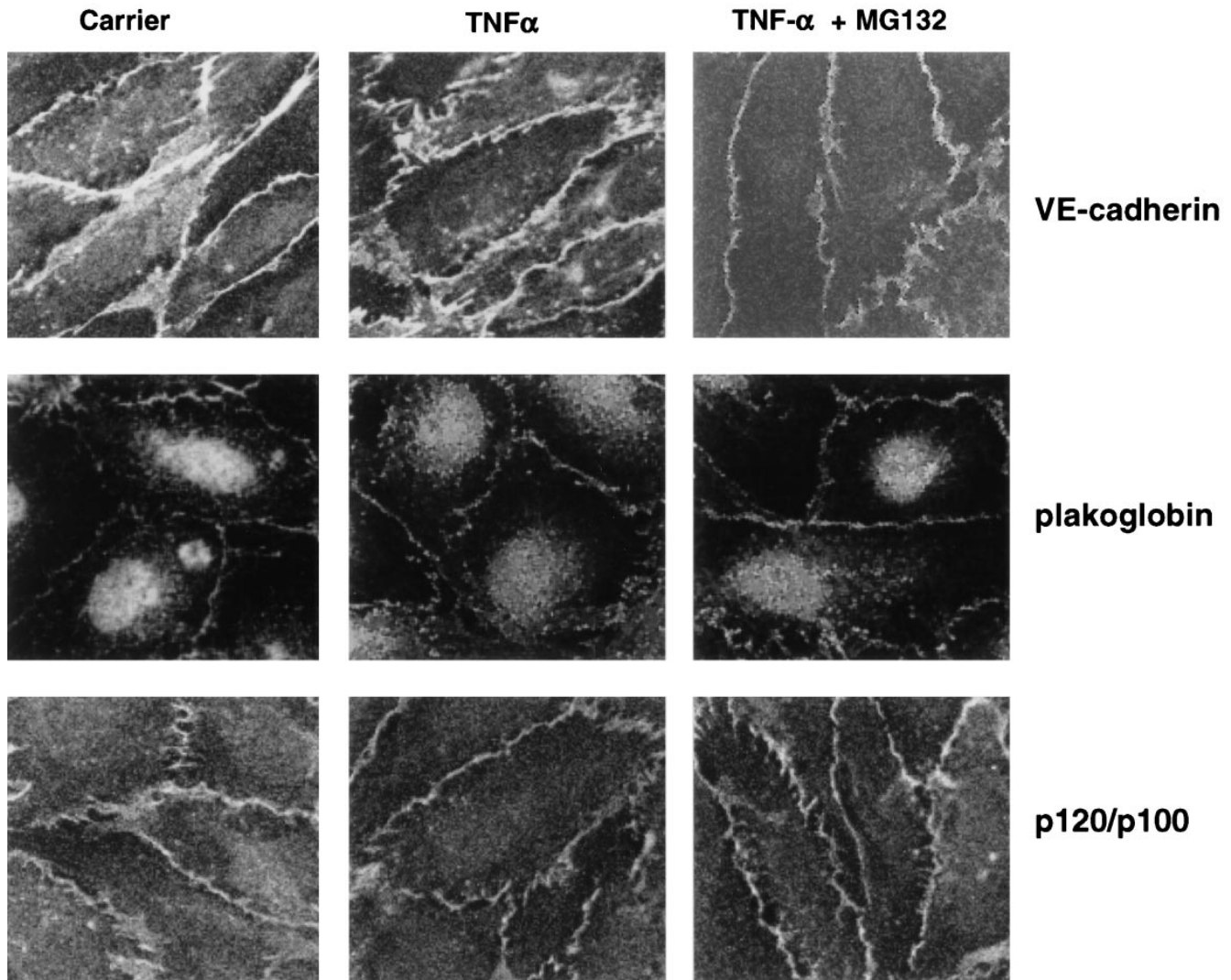


Figure 3. Colocalization of VE-cadherin, plakoglobin, and p120/p100 to lateral cell-to-cell junctions in confluent EC monolayers is not altered by TNF- α alone or TNF- α plus MG132. Confluent cultured EC were treated with carrier (0.02% DMSO), TNF- α (4 h), or TNF- α (4 h) followed by 2 h of incubation with MG132 (20 μ M), washed, and fixed on ice with cold methanol for 5 min. Complex components were detected using specific mAb as detailed in Materials and Methods.

and ALLM; data with lactacystin not shown). Similarly, treatment with TNF- α for 4 h followed by 2 h of coincubation with inhibitors, ALLM, or carrier control did not alter the VE-cadherin complex (data not shown).

Analysis of Triton X-100 cell lysates also revealed that the majority of VE-cadherin and other members of the cadherin complex remained in the Triton X-100-soluble fraction, not in the cytoskeleton-associated Triton X-100-insoluble lysate (data not shown), and this distribution was not altered by any of the above treatments.

Results of the immunoprecipitation and immunoblots were supported by data obtained from indirect immunofluorescence photomicroscopy (Fig. 3). TNF- α , lactacystin, MG132, or ALLM treatments alone, or incubations for 4 h with TNF- α followed by 2 h incubations with either inhibitors or ALLM, had no effect on the localization of the components of VE-cadherin complex to the lateral junc-

tions. The results suggest that treatment of confluent EC monolayers with TNF- α or inhibitors, alone or in combination, does not alter the biochemical composition or lateral junction localization of the VE-cadherin complex.

Neutrophil Adhesion/Transmigration Dramatically Disrupts the VE-Cadherin Complex. To examine whether neutrophil transmigration correlated with any alterations in the VE-cadherin complex, neutrophils were incubated with TNF- α -activated EC monolayers for 10 min, and then the nonadherent neutrophils were removed by washing. Visual inspection of TNF- α -treated EC cultures before lysis showed intact and tightly confluent monolayers with many adherent and transmigrated neutrophils. The ratio of adherent neutrophils to individual EC was 1:2. In contrast, few neutrophils adhered to unactivated EC, and no neutrophils transmigrated. When the levels of VE-cadherin complex were determined in Triton X-100-soluble lysates by im-

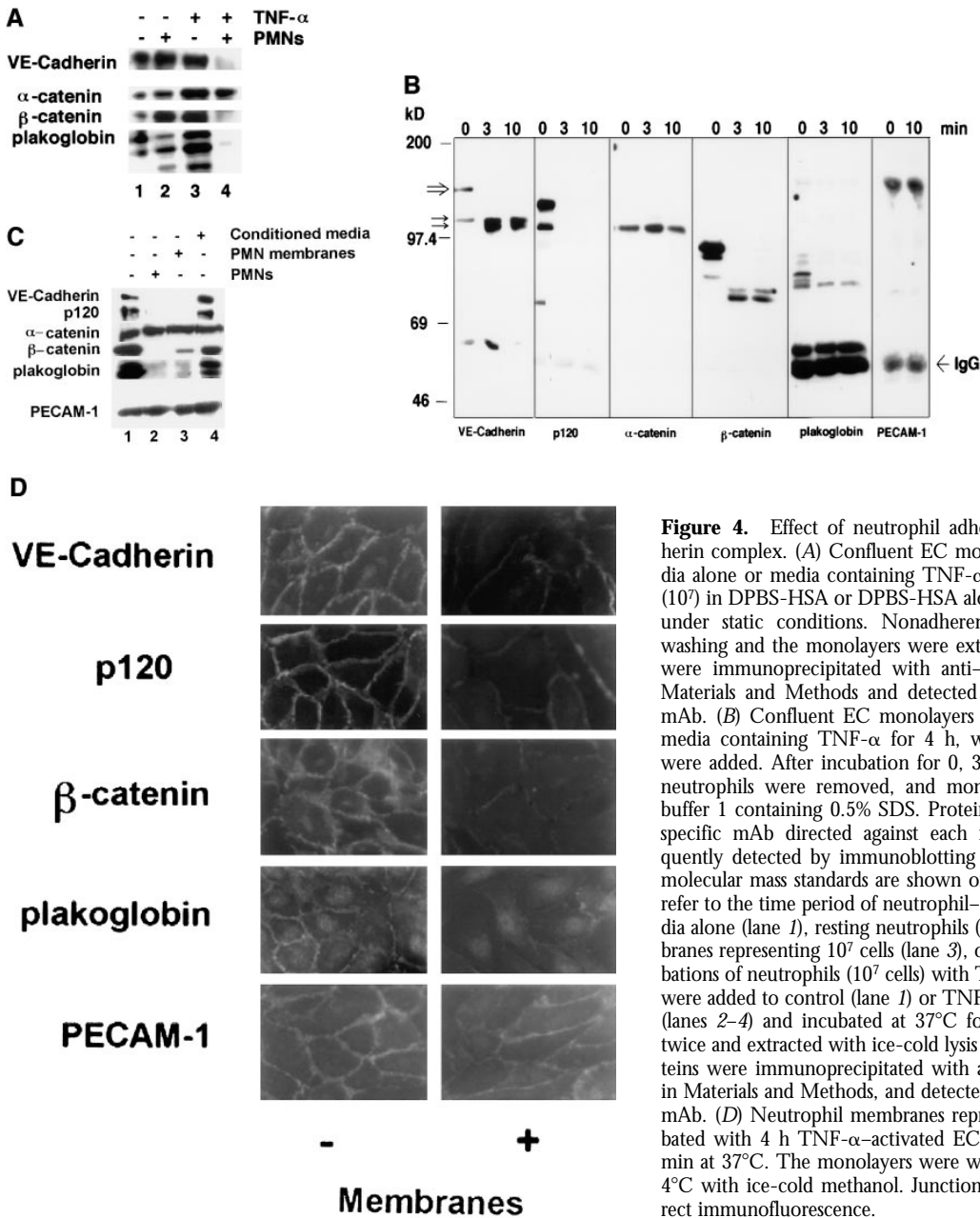


Figure 4. Effect of neutrophil adhesion on the endothelial VE-cadherin complex. (A) Confluent EC monolayers were incubated with media alone or media containing TNF- α for 6 h and washed. Neutrophils (10^7) in DPBS-HSA or DPBS-HSA alone were added for 10 min at 37°C under static conditions. Nonadherent neutrophils were removed by washing and the monolayers were extracted on ice for 30 min. Proteins were immunoprecipitated with anti-VE-cadherin mAb as detailed in Materials and Methods and detected by immunoblotting with specific mAb. (B) Confluent EC monolayers were treated with media alone or media containing TNF- α for 4 h, washed twice, and 10^7 neutrophils were added. After incubation for 0, 3, or 10 min at 37°C, nonadherent neutrophils were removed, and monolayers were extracted with lysis buffer 1 containing 0.5% SDS. Proteins were immunoprecipitated using specific mAb directed against each individual component and subsequently detected by immunoblotting with a specific mAb. The M_r for molecular mass standards are shown on the left margin and 0, 3, and 10 refer to the time period of neutrophil-EC cocubation at 37°C. (C) Media alone (lane 1), resting neutrophils (10^7 cells, lane 2), neutrophil membranes representing 10^7 cells (lane 3), or conditioned media from cocubations of neutrophils (10^7 cells) with TNF- α -activated HUVEC (lane 4) were added to control (lane 1) or TNF- α -activated HUVEC monolayers (lanes 2-4) and incubated at 37°C for 10 min. HUVEC were washed twice and extracted with ice-cold lysis buffer 1 (without 0.5% SDS). Proteins were immunoprecipitated with anti-VE-cadherin mAb as detailed in Materials and Methods, and detected by immunoblotting with specific mAb. (D) Neutrophil membranes representing 3×10^6 cells were incubated with 4 h TNF- α -activated EC in wells of chamber slides for 10 min at 37°C. The monolayers were washed twice and fixed for 5 min at 4°C with ice-cold methanol. Junctional proteins were detected by indirect immunofluorescence.

munoprecipitation with anti-VE-cadherin mAb and immunoblotting with specific mAb to each component, dramatic alterations were observed. Neutrophil adhesion and/or migration across TNF- α -activated EC monolayers induced loss of VE-cadherin, β -catenin, and plakoglobin, whereas the level of α -catenin was not decreased (Fig. 4 A, compare lane 3 to 4). Over the course of our experiments, we noted that the native immunoreactive species of β -catenin, plakoglobin, and p120/p100 were always below detectable levels, whereas there was often retention of a small amount of VE-cadherin. Cocubation of control unactivated EC monolayers with unactivated neutrophils consistently had no significant effect on the VE-cadherin complex (compare

lane 1 and 2). Since equal numbers of EC were used for each sample, and this ECL detection system is very sensitive, we infer the losses are not due to unequal sample loading.

Neutrophil Adhesion Induces Rapid Disruption and Degradation of VE-Cadherin Complex. The disruption of the VE-Cadherin complex by addition of neutrophils was accompanied by significant loss and proteolytic cleavage of each component, except α -catenin. This was addressed by using total EC monolayer lysates (soluble in 1% Triton X-100, 1% NP-40, and 0.5% SDS) and performing immunoprecipitation with specific mAb directed against each component. Immunoprecipitated proteins were detected subse-

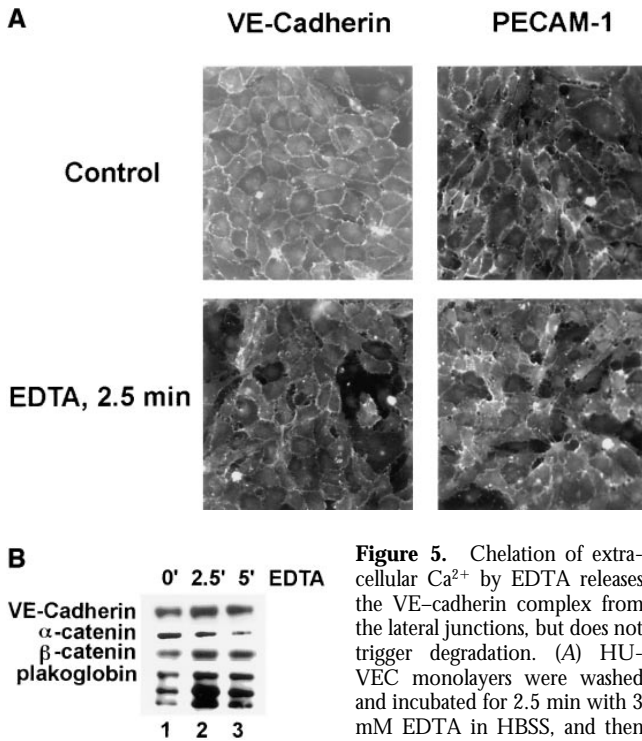


Figure 5. Chelation of extracellular Ca^{2+} by EDTA releases the VE-cadherin complex from the lateral junctions, but does not trigger degradation. (A) HUVEC monolayers were washed and incubated for 2.5 min with 3 mM EDTA in HBSS, and then fixed for 5 min in ice-cold methanol.

VE-cadherin or PECAM-1 was detected by indirect immunofluorescence as detailed in Materials and Methods, and the images were captured using a cooled charged-coupled device camera. (B) Immunoprecipitation and blotting studies show that incubation with EDTA does not induce cleavage of VE-cadherin. Confluent EC monolayers were incubated with $\text{TNF-}\alpha$ for 4 h, and then washed twice with DPBS and incubated in either HBSS (lane 1) or HBSS medium containing 3 mM EDTA for 2.5 (lane 2) or 5 min (lane 3) at 37°C . VE-cadherin was immunoprecipitated, resolved by SDS-PAGE, and subjected to blotting as detailed in Fig. 3 legend.

quently by immunoblotting with the same mAb. Addition of SDS dissociates the complex from the cytoskeletal components (8), and thus allows for determination of the total cellular content (soluble and cytoskeletal-associated) of each component of the complex. Total lysates were prepared from EC treated with $\text{TNF-}\alpha$ and incubated with media alone or media with neutrophils for 0, 3, or 10 min (Fig. 4 B, lanes 0, 3, and 10, respectively, for each mAb listed). In parallel, adhesion and transmigration was assessed by phase contrast microscopy. The levels of native β -catenin, VE-cadherin, plakoglobin, and p120/p100 were decreased dramatically in $\text{TNF-}\alpha$ -activated EC coincubated with neutrophils for 3 min, a time point when neutrophil adhesion, but no transmigration, had occurred. By 10 min, when many neutrophils had adhered and transmigrated, the levels of each component, except α -catenin, was reduced dramatically or undetectable (Fig. 4 B, lanes labeled 10). The level and apparent M_r of α -catenin was stable at each time point. These results are consistent with the immunoblots of the VE-cadherin complex shown in Fig. 4 A. In contrast, PECAM-1, which has been demonstrated to localize to endothelial cell-to-cell lateral junctions and is involved in neutrophil

migration (15), remained at similar levels at both 0 and 10 min.

Inspection of immunoblots of total lysates (Fig. 4 B) from $\text{TNF-}\alpha$ -activated EC coincubated with neutrophils using anti-VE-cadherin mAb revealed the native VE-cadherin species (140 kD, Fig. 4 B, *large arrow*, VE-cadherin, lane 0), and two immunoreactive bands with an apparent M_r of ~ 100 kD (*small arrow*) after 3 or 10 min. That α -catenin remains associated with VE-cadherin, as demonstrated in Fig. 4 A, may be due to its continued association with these 100-kD VE-cadherin immunoreactive degradation products identified in Fig. 4 B. Previous studies have reported that α -catenins can associate directly with VE-cadherin in some cells types (26), but not in others (27). Immunoblots of total lysates with mAb to β -catenin revealed an immunoreactive degradation product at ~ 75 kD, and essentially total loss of immunoreactive bands for p120/p100 and plakoglobin. These data rule out the possibility that components of the VE-cadherin complex shift their distribution from Triton X-100-soluble to -insoluble fraction (i.e., cytoskeletal associated) after neutrophil adhesion. From these findings, we infer that neutrophil adhesion alone is sufficient to induce rapid endothelial-dependent disruption and partial degradation of members of the VE-cadherin complex.

We then performed several experiments to show that neutrophils trigger an endothelial-dependent signal that leads to degradation of specific members of the complex. First, the members of the VE-cadherin complex were clearly disrupted after a 10 min coincubation of $\text{TNF-}\alpha$ -activated EC with neutrophil membranes (representing 10^7 neutrophils, Fig. 4 C, lane 3), which are devoid of granule contents and had only residual elastase activity (0.38% of total elastase activity when compared to intact neutrophils). In addition, disruption of the VE-cadherin complex by incubation of EC monolayers with neutrophil membranes was demonstrated by indirect immunofluorescence studies (Fig. 4 D). Thus, the components that triggered disruption of the VE-cadherin complex are present in neutrophil plasma membranes. This suggests that the degradation is not due to nonspecific degradation via neutrophil granule proteolytic enzymes. Second, transfer of conditioned medium from coincubation of neutrophils with $\text{TNF-}\alpha$ -activated EC to $\text{TNF-}\alpha$ -activated EC did not alter the VE-cadherin complex (Fig. 4 C, lane 4), suggesting degradation is not induced by a soluble factor. Lastly, neutrophil interactions specifically trigger both the disruption and partial degradation of VE-cadherin complex since incubation of EC monolayers with EDTA, which released VE-cadherin from the lateral junctions (Fig. 5 A), did not trigger degradation of VE-cadherin complex (Fig. 5 B). Taken together, these findings suggest that the dissociation and cleavage of VE-cadherin and its associated proteins are carefully controlled endothelial-specific event(s) that are triggered by leukocyte contact.

Indirect immunofluorescence photomicroscopy supported the findings that neutrophil adhesion induced loss of the VE-cadherin complex at EC lateral junctions (Fig. 6; neutrophils are identified with arrows). $\text{TNF-}\alpha$ -activated EC monolayers were incubated with or without human neu-

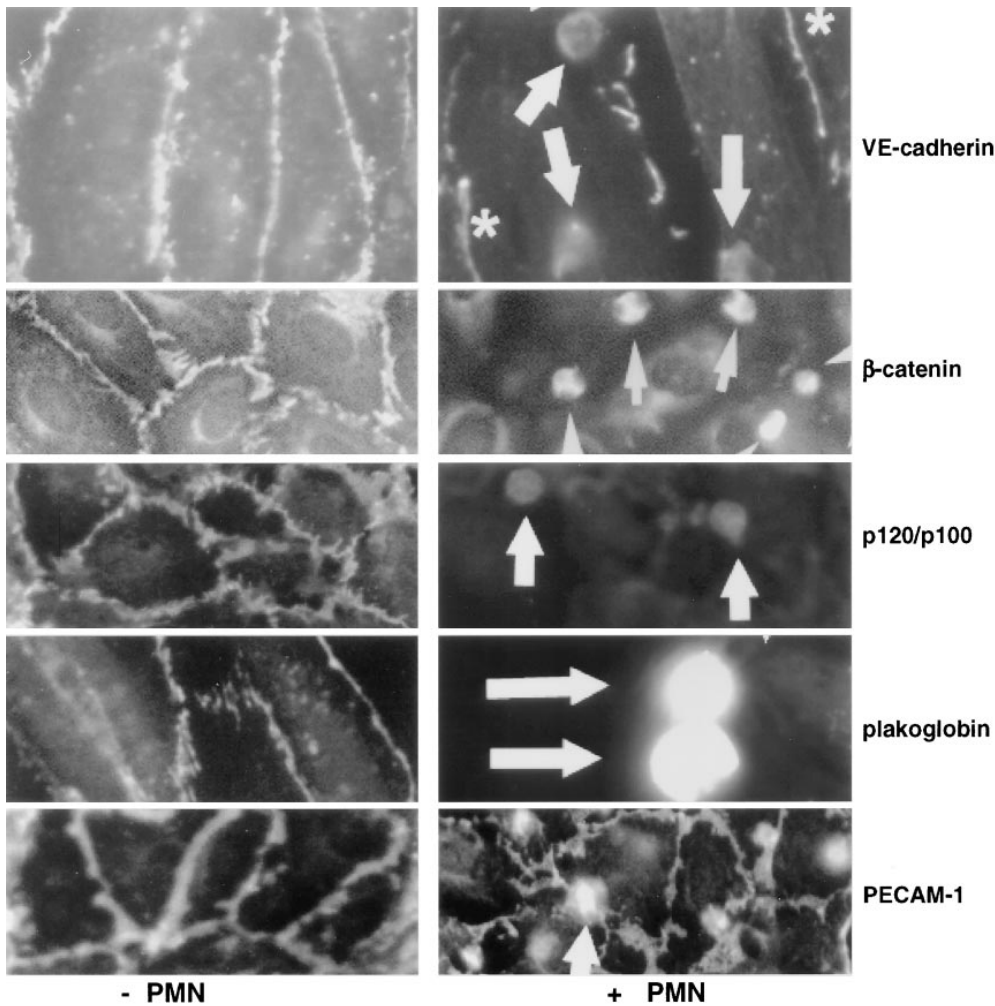


Figure 6. Photographs show VE-cadherin, β -catenin, p120/p100, plakoglobin, and PECAM-1 staining patterns. Indirect immunofluorescence microscopy shows that TNF- α -activated EC monolayers express abundant levels of VE-cadherin, β -catenin, p120/p100, plakoglobin, and PECAM-1 at lateral junctions before neutrophil adhesion (*left*). After 10 min of neutrophil adhesion, the lateral staining of VE-cadherin, β -catenin, p120/p100, and plakoglobin is lost, whereas PECAM-1 is still abundantly expressed at lateral junctions (*right*). Adherent neutrophils are identified (*right, arrows*), and intact VE-cadherin junctional staining (*top asterisks*). Original magnification was 320, except VE-cadherin which was 640.

trophils for 5 min, fixed, and then stained with mAb to the VE-cadherin complex. The loss is clearly demonstrated for monolayers stained to detect VE-cadherin, β -catenin, p120/p100, and plakoglobin, whereas the level and junctional colocalization of PECAM-1 remained constant. A significant overall reduction in immunoreactive staining of β -catenin, p120/p100, and plakoglobin was observed in the presence of neutrophils, whereas VE-cadherin was lost specifically from regions of neutrophil adhesion/transmigration (*small arrows*). This is consistent with the immunoprecipitation data where, in the majority of experiments, a small amount of native VE-cadherin is retained, whereas other components of the complex are not detectable.

To investigate whether the degradation of the VE-cadherin complex involved the cellular proteasome system, the effects of proteasome inhibitors were evaluated. As shown in Fig. 7, disruption of the VE-cadherin complex was not prevented by 2 h of incubation with 20 μ M lactacystin or MG132, even though such treatments significantly reduced transmigration (Fig. 1). For comparison, the components of the VE-cadherin complex in TNF- α -activated EC remained constant in the absence of neutrophils. In addition, shorter incubations (5 or 30 min) with 50 μ M MG132 did

not prevent neutrophil-dependent dissociation of the VE-cadherin complex (data not shown). These findings establish that neutrophil adhesion triggers rapid EC-dependent degradation of the VE-cadherin complex through an enzymatic pathway that appears to be independent of the proteasome.

Discussion

The current work demonstrates that neutrophil adhesion to TNF- α -activated endothelial monolayers dramatically alters the molecular composition and organization of the endothelial cell VE-cadherin complex, which has been implicated in maintenance of endothelial cell-to-cell adhesion and cell-to-cytoskeletal integrity. Neutrophil adhesion to confluent TNF- α -activated endothelial monolayers induced rapid and near complete dissociation of β -catenin, p120/p100, and plakoglobin from VE-cadherin and loss of their lateral junction colocalization. Biochemical analysis of TNF- α -activated EC monolayers using immunoprecipitation and immunoblotting revealed that these proteins are degraded rapidly through a proteolytic mechanism that is dependent on the endothelium and does not involve gran-

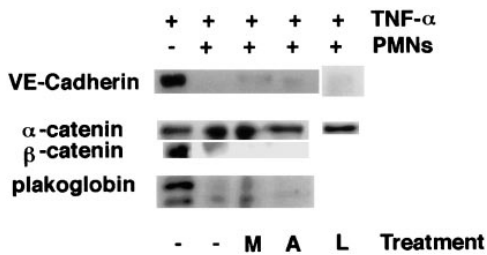


Figure 7. Proteasome inhibitors do not prevent neutrophil-induced disruption of the VE-cadherin complex. Neutrophils (10^7 in 5 ml) were incubated with EC monolayers that were treated with TNF- α (4 h, 25 ng/ml) followed by 2 h of incubation with MG132 (M), ALLM (A), or lactacystin (L), or 0.02% DMSO (-). After 10 min at 37°C, EC monolayers were extracted with lysis buffer 1 and processed for detection of the VE-cadherin complex.

ule components of the neutrophil. In contrast, PECAM-1, which is not associated directly with the VE-cadherin complex, but does colocalize to the lateral EC junctions and is involved in leukocyte transmigration (28), remained at constant levels and retained lateral junction localization. Given the rapid and specific cleavage of the components of VE-cadherin complex by the endothelial cells, we suspected that the cellular proteasome was involved. Surprisingly, two different proteasome inhibitors, MG132 or lactacystin, did not prevent dissociation or degradation of components of the VE-cadherin complex, however, both inhibitors did prevent >60% of neutrophil transmigration, specifically by preventing neutrophil penetration of the lateral junctions. Taken together, the results demonstrate that leukocyte adhesion and transmigration across the vascular endothelium is more complex than previously appreciated, and that neutrophil adhesion induces substantial alterations in structural components of the adherens junctions that may allow for subsequent transmigration of neutrophils.

The current finding that neutrophil coincubation with TNF- α -activated EC disrupts the VE-cadherin complex as early as 3 min demonstrates that neutrophil-EC adhesion, rather than transmigration, is the initiating signal. As reported in the text, visual inspection of monolayers consistently showed that no transmigration had occurred at 3 min, even though disruption and degradation of the VE-cadherin complex was easily detected (Fig. 4 B). These data suggest engagement of adhesion molecules may be crucial in triggering EC-dependent events, although signaling through molecules not involved in adhesion is certainly possible. This notion is further supported by the positive effects of neutrophil membranes on the VE-cadherin complex. Additional studies are necessary to elucidate the intracellular signaling mechanisms underlying VE-cadherin complex alterations.

The observed changes in the association of VE-cadherin with cytosolic α -catenin, β -catenin, and plakoglobin after leukocyte adhesion/transmigration is interesting in a few respects. Using conditions to retain intact VE-cadherin complex, α -catenin remains associated with the \sim 100-kD fragments of VE-cadherin, whereas β -catenin and plakoglobin

do not. This is somewhat unexpected since α -catenin does not appear to associate directly with members of the cadherin family in most cells examined (29, 30). One explanation is that the cleaved \sim 100-kD fragments of VE-cadherin can associate directly with α -catenin, even though VE-cadherin is not localized at the lateral junction. This is not unreasonable since α -catenin has been reported recently to stably associate with VE-cadherin in EC and in Chinese hamster ovary cells transiently transfected with VE-cadherin (26). Secondly, the immunoprecipitation and blotting analyses strongly suggest that p120/p100 associates with the VE-cadherin complex in confluent EC monolayers. Although the association of p120/p100 with VE-cadherin complex was not observed in every EC culture, direct immunoprecipitation from total EC extracts and immunoblotting with anti-p120 mAb consistently detected two specific bands at 120 and 100 kD, which is in line with the report of Staddon and co-workers (31). Moreover, both biochemical and indirect immunofluorescence evidence showed that neutrophil coincubation with activated EC rapidly led to the degradation and loss of lateral junctional staining of p120/p100. One possible explanation for the inconsistency of immunoprecipitation experiments using anti-VE-cadherin mAb is that only a small fraction of the cytosolic pool of p120/p100 associates with VE-cadherin, or that the association fluctuates due to tissue culture conditions and thus the signal is low relative to the VE-cadherin complex signal. Staddon et al. (12) have reported that in Mardin-Darby canine kidney epithelial cells, only 20% of the cytosolic pool of p120/p100 associates with β -catenin.

While this manuscript was being reviewed, Del Maschio et al. (32) reported a similar effect of neutrophils on the VE-cadherin complex in human EC monolayers. The current work confirms that neutrophils induce rapid adhesion-dependent alterations in the VE-cadherin complex, and extends these observations to show that (a) disruption and degradation is endothelial-dependent and independent of neutrophil proteolysis and (b) independent of the endothelial proteasome system. Interestingly, Del Maschio et al. described significant retention of VE-cadherin while still detecting dramatic loss of β -catenin and plakoglobin (essentially equivalent to the data reported in Fig. 4). The reason for this difference in the level of VE-cadherin retained at EC junctions is not clear. It cannot be explained by a difference in buffers because we still detect a dramatic loss of VE-cadherin and its components when using their buffer system for neutrophil-EC coincubations (M199 with or without 20% FCS), lysis, and immunoprecipitation.

The proteasome may play an important role in regulating endothelial-leukocyte interactions. If selective proteolysis lies at the center of a diverse array of cellular processes, then a question is what controls its timing. Resolving these issues may be important in understanding the role of the proteasome in leukocyte transmigration. How the proteasomal inhibitors interfere with neutrophil transmigration is not clear. Several possibilities can be considered. First, specific signal transduction events initiated by endothelial-leukocyte interactions may require the ubiquitin-proteasome

proteolytic pathway. The paradigm for this is well established for several processes (13, 33). Second, the proteasome may degrade key architectural components of the junctional complex. The VE-cadherin complex is probably not the target of these events. Endothelial-leukocyte interactions could alter the activity of the endothelial proteasome by altering the composition or by increasing the activity of specific catalytic components of the proteasome facilitating transmigration. Third, the proteasome inhibitors may act indirectly by altering the activity of important processes other than the proteasome. The structure-function relationship between the inhibitors used in this study suggests that this possibility is unlikely. Two structurally distinct proteasome inhibitors, lactacystin and MG132, specifically blocked leukocyte transmigration. A structurally homologous peptide aldehyde (ALLM), which inhibits cathepsin B and calpain but is a much weaker inhibitor of the proteasome, did not block transmigration. Collectively, these findings distinguish the proteasome pathway rather than cathepsin/calpain-mediated protein degradation or processing as an important step in leukocyte transmigration.

The Process of Leukocyte Transendothelial Migration. The current report, and several previous studies, further expand the notion that EC-leukocyte adhesion triggers EC-dependent changes that correlate with leukocyte transmigration. Huang et al. (34) showed that intracellular Ca^{2+} gradients in EC were coupled to transendothelial migration. Neutrophil adhesion to EC induced rapid and transient several fold increases in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$), which paralleled the time course of neutrophil transmigration across cytokine-activated EC grown on human amnion prepara-

tions. Pharmacological clamping of $[Ca^{2+}]_i$ with an intracellular Ca^{2+} chelator, bis-(2-amino-5-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester, inhibited >90% of migration, but had no effect on adhesion. Pfau et al., working with lymphocytes and EC (35), and Zeigelstein et al., using both neutrophils and monocytes interacting with endothelial monolayers under flow conditions in vitro (36), also reported leukocyte adhesion-dependent changes of $[Ca^{2+}]_i$ in EC and the leukocyte. Neither of these studies report on the spatial resolution of the Ca^{2+} flux, so it remains to be determined whether the cationic flux relates to an adhesion event or to a migratory event. Thus, the role of increases of $[Ca^{2+}]_i$ in EC appear compelling, whereas the relative role in leukocytes is less clear. Interestingly, in a static assay system, pharmacologic clamping of $[Ca^{2+}]_i$ in neutrophils did not dramatically alter their migration across resting or cytokine-activated EC, suggesting that changes in neutrophils $[Ca^{2+}]_i$ are not a prerequisite (37). In addition, Yoshida et al. (31) have reported that binding of leukocytes to 4-h IL-1-activated EC induce E-selectin linkage at its cytoplasmic domain to the EC actin cytoskeleton. This link, or similar mechanisms, may supply cues to the EC that influence downstream processes involved in stable adhesion or in transmigration.

The results presented here raise several issues concerning the function of lateral junctions during leukocyte trafficking at sites of inflammation. A basic understanding of these processes may lead to insight into therapies for prevention of tissue damage and abnormal wound healing that occur as a result of a pathological inflammatory response.

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Address correspondence to Dr. Francis W. Luscinskas, Brigham and Women's Hospital, 221 Longwood Ave., Boston, MA 02115. Phone: 617-732-6004; FAX 617-732-5933; E-mail: fluscinska@bics.bwh.harvard.edu

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