In Vitro Degradation of Endothelial Catenins by a Neutrophil Protease

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Abstract. It has been recently proposed that adhesion of polymorphonuclear cells (PMNs) to human umbilical vein endothelial cells leads to the disorganization of the vascular endothelial cadherin–dependent endothelial adherens junctions. Combined immunofluorescence and biochemical data suggested that after adhesion of PMNs to the endothelial cell surface, β-catenin, as well as plakoglobin was lost from the cadherin/catenin complex and from total cell lysates. In this study we present data that strongly suggest that the adhesion-dependent disappearance of endothelial catenins is not mediated by a leukocyte to endothelium signaling event, but is due to the activity of a neutrophil protease that is released upon detergent lysis of the cells.

The endothelium forms the main barrier that, under homeostatic conditions, regulates the diffusion and transport of both macromolecules and whole cells from the blood stream to the underlying tissues. In response to an inflammatory stimulus, polymorphonuclear leukocytes (PMNs)[1] are the first cells that are recruited from the blood to the site of an acute inflammatory reaction. This extravasation process is initiated by a cascade of cell adhesion molecules and leukocyte-activating mediators, which control the adhesion of leukocytes to the apical surface of endothelial cells (EC) (Carlos and Harlan, 1994; Springer, 1994).

Whereas these initial interactions have been intensively studied, the ensuing transmigration event is poorly understood. Transendothelial migration requires mechanisms that open the endothelial cell layer and allow the passage of leukocytes. Endothelial monolayer integrity and permeability, on the other hand, are largely controlled by intercellular junctions (Rubin, 1992; Dejana et al., 1995). With respect to leukocyte extravasation, the so-called adherens junctions appear to be of particular interest. These junctions are formed by the cadherins, transmembranous cell–cell adhesion molecules that undergo homophilic interactions and that bind to each other in a Ca^{2+}-dependent manner. To perform their adhesive functions, these cadherins interact with the actin cytoskeleton through their cytoplasmic tails, an association that is mediated by the intracellular catenins α-catenin, β-catenin, and plakoglobin (Takeichi, 1991; Kemler, 1993; Aberle et al., 1996). In the endothelium, several cadherins have been described, of which only vascular endothelial (VE)-cadherin (cadherin-5) is specific for endothelial cells (Liaw et al., 1990; Suzuki et al., 1991; Lampugnani et al., 1992). VE-cadherin is concentrated at sites of cell–cell contacts, and functions in the maintenance of cell layer integrity of cultured human endothelial cells (Lampugnani et al., 1992; Navarro et al., 1995). A monoclonal antibody against mouse VE-cadherin accelerates the extravasation of neutrophils in a mouse peritonitis model in vivo (Gotsch et al., 1997), suggesting that the opening of VE-cadherin–mediated cell contacts may be a relevant step during neutrophil extravasation. Whereas the mechanisms that would lead to such an opening of adherens junctions have not been defined, it has nevertheless been demonstrated that adhesion of PMNs leads to an increase in endothelial cytosolic Ca^{2+} levels. In addition, intracellular Ca^{2+} scavengers were shown to block PMN transmigration (Huang et al., 1993).

Based on this, Del Maschio et al. (1996) have recently presented evidence that suggested that PMN adhesion would trigger the disorganization of endothelial adherens junctions. By using immunofluorescence as well as immunoprecipitation and Western blotting techniques, the authors found that the VE-cadherin/catenin constituents of adherens junctions disappeared from the endothelial cell–cell contacts. In addition, and even more surprising, β-catenin as well as plakoglobin completely disappeared from total cell extracts, suggesting that PMN adhesion would lead to the activation of a catenin-degrading proteolytic activity

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1. *Abbreviations used in this paper*: EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; PFA, paraformaldehyde; PMN, polymorphonuclear cell; VE, vascular endothelial.

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(Del Maschio et al., 1996). Similar results were recently also described by Allport et al. (1997), who extend the above observations by showing that the endothelial proteasome is not involved in catenin degradation. Here, we present evidence that strongly suggests that this catenin-degrading activity is not an endothelial enzyme but leukocyte encoded. Our data lead us to conclude that the disappearance of catenins after the adhesion of PMNs to EC (and seen in immunofluorescence as well as by Western blotting of total cell lysates), is because of a nonspecific proteolytic event.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated as described (Warren, 1990), and cultured in M199, 20% FCS, 50 μg/ml endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO), 100 μg/ml Heparin (Sigma Chemical Co.). Alternatively, HUVEC were purchased commercially (Promocell, Heidelberg, Germany) and cultivated in MCDB 131 (GIBCO BRL, Gaithersburg, MD), 20% FCS, 50 μg/ml ECGS, 100 μg/ml Heparin. Cells were used up to passage number 8. Mouse bEnd.3 endothelioma cells (Williams et al., 1989) were cultured as described (Hahne et al., 1993). Activation of the EC was done with 400 U/ml tumor necrosis factor (TNF)-α for 4 h or 24 h as indicated.

Leukocyte Adhesion to EC

Human PMN were isolated from fresh buffy coats on Histopaque 1077 and Histopaque 1119 density gradients according to the manufacturer's instructions (Sigma Chemical Co.). The recovered cells were washed twice in HBSS without Ca^{2+} and Mg^{2+} (GIBCO BRL, Gaithersburg, MD) and kept in this buffer at room temperature until use. The leukocytes were then resuspended into M199, 20% FCS (for HUVEC) or DME, 10% FCS (for bEnd.3), and then added to the EC at a 10:1 or 2:1 ratio of leukocytes to EC as indicated. The cells were coincubated for 5 min at 37°C in a humidified atmosphere. Nonadherent cells were washed off with PBS without Ca^{2+} and Mg^{2+}, and the remaining cells were lysed. Lysis was carried out in either lysis buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM Tris/HCl, pH 8.0, 1 mM $CaCl_2$, 10 μg/ml leupeptin, 1 mM PMSF, 2 μg/ml pepstatin, 40 U/ml aprotinin, 30 μg/ml eglin C, or in boiling (95°C) Laemmli SDS-PAGE sample buffer (2% SDS, 10% glycerol, 65 mM Tris/HCl, pH 6.8, 50 mM DTT, [Laemmli, 1970]). For Triton X-100 lysates, lysis was carried out on the tissue culture plates for 25 min on ice with occasional gentle agitation. Cell extracts were then centrifuged at 14,000 rpm in a table top microfuge for 5 min at 4°C, and the supernatants used (Triton X-100–soluble fraction). The residual material on the tissue culture plate was extracted with 0.2% SDS, briefly sonicated, and then analyzed (Triton X-100–insoluble fraction; data not shown). In some instances, after the rinse in PBS, the adherent PMNs were removed by incubation with PBS without Ca^{2+} and Mg^{2+}, 5 mM EDTA for about 30 s. Subsequently, the PMNs were washed off once more with PBS before extraction with Triton X-100 lysis buffer. For the lysate mixing experiment, Triton X-100 lysates of 4×10^5 HUVEC each were prepared as described above. To such lysates, 2×10^6 lysed PMNs, or mononuclear cells (without centrifugation), or the respective cleared PMN lysates (with nuclei and Triton X-100–insoluble material pelleted) were added as described in the text. Under the experimental conditions used, i.e., PMN/EC ratio of 10:1, we found that approximately five PMNs adhere per endothelial cell. The final volume (300 μl) was the same as for lysates prepared on cell culture plates.

Western Blot Analysis

Cell extracts corresponding to 6×10^4 EC/lane were used and the Western blots performed essentially as described (Zöllner and Vestweber, 1996). To load approximately the same amount of PMN lysates in the lanes where PMNs were analyzed for their respective catenin and α-actinin expression levels, lysates corresponding to 3×10^5 cells were used (see above). For detection of the human antigens monoclonal antibodies against β-catenin (No. C19220) and plakoglobin (No. C26220; all from Transduction Laboratories, Lexington, KY), and against α-actinin (BM-75.2; Sigma Chemical Co.) were used according to the manufacturer's instructions.

Immunofluorescence Microscopy

Immunofluorescence analysis was performed essentially as described (Lampugnani et al., 1992; Del Maschio et al., 1996), except that endothelial cells were grown on coverslips coated with 0.2% gelatine. The removal of adherent PMN with EDTA was carried out as described above. Cells were fixed in 3% paraformaldehyde (PFA) in PBS; 0.5% Triton X-100 for 3 min at room temperature, followed by 3% PFA in PBS for 15 min at room temperature. Identical results were obtained when cells were first fixed with 3% PFA for 10 min at room temperature, and subsequently permeabilized with 0.5% Triton X-100 in HBSS for an additional 5 min. Mouse VE-cadherin and α-catenin were detected using rabbit polyclonal antipeptide antibodies (5 μg/ml in HBSS, 3% FCS) and have been described (Breier et al., 1996; Gotsch et al., 1997). β-catenin was detected using a monoclonal antibody (1:100 in HBSS, 3% FCS; No. C19220) followed by a TRITC-conjugated, goat anti–mouse secondary antibody (1:50 in HBSS, 3% FCS; Dianova, Hamburg, Germany).

Results and Discussion

Neutrophil-induced Catenin Disappearance Depends on Cell Lysis Conditions

Recent work by Del Maschio et al. (1996) suggested that PMN adhesion to EC leads to the complete loss of β-catenin and plakoglobin from total cell extracts, as well as to an at least partial disappearance of VE-cadherin and α-catenin. We could reproduce this effect by adding human PMNs for 5 min to monolayers of 4 h TNF-α–stimulated HUVEC, washing unbound cells away, lysing endothelial cells and bound neutrophils with Triton X-100 (in the presence of a cocktail of protease inhibitors), and immunoblotting the extracts with antibodies to β-catenin and plakoglobin (Fig. 1 *A*, lanes *3* and *4*). The same effect was seen with HUVEC activated for 24 h (data not shown). β-catenin and plakoglobin also disappeared from the Triton X-100–insoluble fraction, which was solubilized by SDS (data not shown).

Since neutrophils are known to produce a large number of proteases, we were concerned as to whether the colysis of EC and bound PMNs in a Triton X-100–containing buffer could possibly lead to the nonspecific liberation of neutrophil proteases—sufficiently active even in the presence of protease inhibitors. Therefore, we changed the lysis conditions and extracted HUVEC and bound neutrophils with boiling (95°C) SDS-PAGE sample buffer. Subsequent immunoblot analysis revealed that β-catenin as well as plakoglobin were not degraded if cells were lysed under these conditions (Fig. 1 *A*, lanes *1* and *2*). These results strongly suggest that degradation of catenins does not occur before cells are lysed and depends on the lysis conditions.

To rule out a potential contribution of neutrophil proteases in the degradation of the catenins Del Maschio et al. (1996) separately extracted HUVEC and PMNs with Triton X-100, pelleted the insoluble material, and then coincubated the detergent extracts on ice. This treatment did not result in any degradation of either VE-cadherin or the catenins. Since the preparation of Triton X-100 lysates and the centrifugation of insoluble material takes about 30 min, we analyzed whether the suspected proteolytic activity in the PMN lysates would be unstable. We prepared Tri-

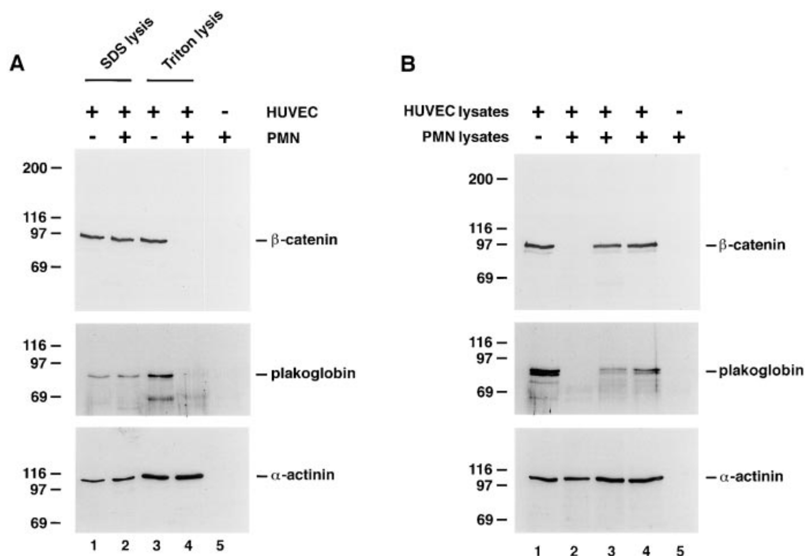

Figure 1. Neutrophil-induced catenin disappearance depends on cell lysis conditions. (*A*) Catenin degradation is inhibited by cell lysis in SDS. HUVEC were stimulated with TNF-α for 4 h (lanes *1–4*). PMNs (PMN/EC ratio 10:1) were added to the HUVEC and incubated at 37°C for 5 min as indicated (lanes *2* and *4*). Nonadherent cells were washed off with PBS, and the remaining cells lysed with boiling (95°C) SDS-PAGE sample buffer (lanes *1* and *2*) or with Triton X-100 lysis buffer (lanes *3* and *4*). The total lysates were probed for β-catenin and plakoglobin by Western blotting. To ensure for equal loading of protein in the respective lanes, the same lysates were also probed for α-actinin (*bottom*). (*B*) The polymorphonuclear cell proteolytic activity is unstable in cell lysates. Triton X-100 cell lysates were prepared from nonstimulated HUVEC. Subsequently, PMN lysates (corresponding to a 5:1 ratio of leukocytes to HUVEC) were resuspended in Triton X-100 lysis buffer. The crude leukocyte lysates either were added to the HUVEC lysates directly, mimicking colysis conditions as seen in adhesion experiments (lane *2*), or incubated on ice for 30 min before adding them to the HUVEC lysates (lane *3*). Alternatively, the PMN lysates were incubated on ice for 25 min and cleared by centrifugation for 5 min before adding them to the EC lysates (lane *4*; mixing conditions as used by Del Maschio et al. [1996]). The mixtures were incubated on ice for an additional 20 min, and subsequently analyzed by immunoblotting for β-catenin, plakoglobin, and α-actinin (as indicated). PMNs alone (*A* and *B*, lane *5*) were assayed for expression of the respective proteins. Molecular weight markers (in kD) are given to the left.

ton X-100 extracts from HUVEC, cleared from insoluble material, and then added PMN lysates prepared in three different ways. First, PMNs were lysed and the total lysate was immediately added to the HUVEC lysate without prior pelleting (Fig. 1 *B*, lane *2*). Second, the total PMN lysate was incubated for 30 min on ice before adding it to the HUVEC lysate, again without prior precipitation of insoluble material (Fig. 1 *B*, lane *3*). Third, PMN lysates were incubated for 30 min on ice, cleared from insoluble material by centrifugation, and then added to the HUVEC lysate (Fig. 1 *B*, lane *4*). The two lysates were coincubated for another 20 min on ice, and then analyzed by immunoblotting. As shown in Fig. 1 *B*, fresh PMN lysates contained a proteolytic activity that degraded β-catenin and plakoglobin (lane *2*). This activity was strongly reduced after a 30-min incubation on ice. Plakoglobin appeared to be more protease sensitive than β-catenin. Interestingly, we found that the neutrophil protease activity was even lost within 30 min if no external protease inhibitors were added (data not shown). Thus, neutrophil lysates degrade endothelial catenins only when immediately added to endothelial lysates.

Removal of Bound Neutrophils with EDTA Before Analysis Prevents Catenin Degradation

We examined whether the disappearance of the endothelial catenins would still be observed if neutrophils that had attached to the endothelial surface were removed by washing them off with EDTA before the analysis. As shown in Fig. 2 the PMN-induced β-catenin and plakoglobin degradation (lane *4*) was largely prevented by washing off the PMNs before extracting the EC with Triton X-100 (lane *6*). Thus, the binding of neutrophils to the endothelial cells was not responsible for the degradation of the catenins, but rather the presence of neutrophils during the lysis procedure.

In addition to the biochemical techniques, immunofluorescence analysis had been used to study the effects of neutrophil binding on the distribution of the catenins at endothelial cell contact sites (Del Maschio et al., 1996). We used the same approach as decribed above (Fig. 2) to control these experiments, i.e., previously bound neutrophils were removed from the endothelial cell monolayer with EDTA before fixation and staining for the endothelial β-catenin. Since the integrity of HUVEC monolayers was very sensitive to the treatment with EDTA, we used

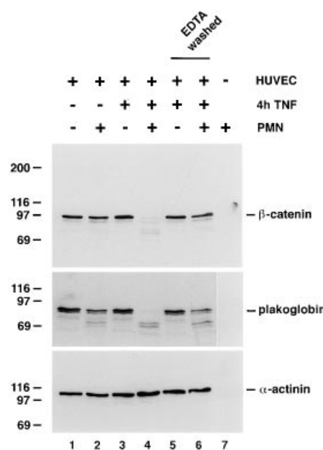

Figure 2. β-catenin and plakoglobin degradation is inhibited by removal of adherent PMNs. HUVEC were either left untreated (lanes *1* and *2*) or stimulated with TNF-α for 4 h (lanes *3–6*), respectively. PMNs (PMN/EC ratio 2:1) were added to the HUVEC and incubated at 37°C for 5 min as indicated (lanes *2*, *4*, and *6*). Nonadherent cells were washed off with PBS, and adherent PMNs were removed in some cases by incubation with 5 mM EDTA for 30 s as indicated (lanes *5* and *6*). Triton X-100 lysates were prepared and probed for β-catenin, plakoglobin, and α-actinin by Western blotting. PMNs alone were assayed for expression of the respective proteins (lane *7*). Molecular weight markers (in kD) are given to the left.

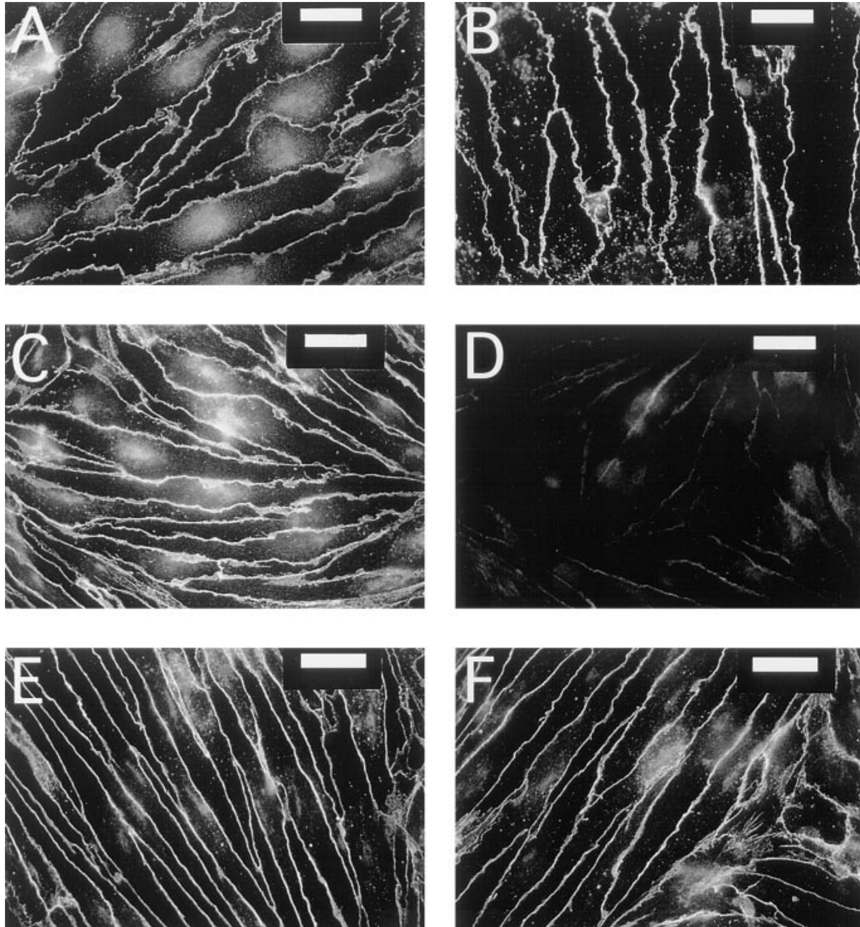

Figure 3. PMN-dependent loss of β-catenin junctional staining is abolished by removal of the PMNs. α-Catenin (*A*), VE-cadherin (*B*), and β-catenin (*C–F*) were visualized in the mouse endothelioma cell line bEnd.3 by indirect in situ immunofluorescence. The EC were stimulated with TNF-α for 4 h, and PMNs (PMN/EC ratio 20:1) were added to the bEnd.3, and then incubated at 37°C for 5 min (*D* and *F*). Bound PMNs were removed by incubating the EC for 30 s with 5 mM EDTA (*F*), as a control the same EDTA treatment was done with EC, to which no PMNs had been added (*E*). Subsequently, the cells were fixed, permeabilized, and the respective proteins visualized as described (see Materials and Methods). Bar, 20 μm.

the mouse endothelioma cell line bEnd.3 for these experiments. bEnd.3 cells not only express VE-cadherin and catenins, but also form functional adherens junctions (Breier et al., 1996; Gotsch et al., 1997) (Fig. 3, *A–C*).

We found that human PMNs bound well to TNFα-stimulated bEnd.3 cells. After the removal of unbound neutrophils, bEnd.3 cells with the bound PMN were fixed with PFA and stained after permeabilization for β-catenin. As shown in Fig. 3 *D*, β-catenin staining was almost completely gone at intercellular contacts. However, removal of specifically bound cells by washing with EDTA before fixation and permeabilization of the monolayer in Triton X-100 completely prevented the disappearance of β-catenin (Fig. 3 *F*). Thus permeabilization of endothelial cells in the presence of adhering PMNs can lead to a neutrophil protease–mediated degradation of β-catenin—despite fixation of the cells with PFA.

Concluding Remarks

In this study we have examined the mechanism by which the binding of neutrophils to the monolayer of endothelial cells causes the degradation of endothelial catenins. In contrast to previous interpretations (Del Maschio et al., 1996; Allport et al., 1997), we found that the catenin-degrading protease in this experimental setting is not an endothelial enzyme, but a leukocyte enzyme that is released upon detergent lysis of the cells. This is based on the following evidence. First, catenin degradation was only observed when the endothelial cells and bound PMNs were detergent lysed under nondenaturing conditions. If the cells were lysed at 95°C in an SDS-containing buffer, degradation was not detected. Second, if PMNs were first allowed to bind to activated HUVEC for 5 min at 37°C, and were then removed by EDTA before detergent lysis of the EC, degradation of catenins was not observed. Third, mixing experiments with detergent extracts of EC and PMNs revealed that catenin disappearance could be observed after mixing extracts from quiescent cells on ice. PMN lysates lost this activity upon incubation on ice for 30 min. This strongly suggests that the PMN-induced endothelial catenin disappearance is not mediated by a transmembrane signaling event, but is because of a neutrophil protease that is released upon detergent lysis.

The nature of the neutrophil protease degrading the endothelial catenins remains obscure. Our data suggest that the activity of this enzyme is not immediately destroyed by 3% PFA. Furthermore, a mixture of several protease inhibitors (see Materials and Methods) did not inactivate the enzyme, and only boiling in 2% SDS efficiently and rapidly destroyed this protease activity. Moreover, several proteins were unaffected by this protease, such as platelet/endothelium (PE)CAM-1 and F-actin (Del Maschio et al., 1996), as well as α-actinin (this study). This may explain

why this neutrophil protease activity was overlooked in the former study.

It has been shown that the binding of PMNs to the apical surface of HUVEC increased the permeability of the endothelial monolayer (Del Maschio et al., 1996), and leads to an increase in endothelial Ca^{2+} levels (Huang et al., 1993). The VE-cadherin/catenin complex is important for the junctional integrity of EC layers (Navarro et al., 1995; Gotsch et al., 1997). Therefore, the concept of adhering leukocytes having an effect on the VE-cadherin junctional complex still remains attractive, although evidence is still lacking. To establish a potential signaling pathway in endothelial cells that connects the docking of PMN at the apical surface to the regulation of VE-cadherin/catenin function, one would have to efficiently inhibit the neutrophil proteolytic activity. Alternatively, one would have to find means to manipulate such a signaling pathway independently of neutrophil adhesion.

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