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) 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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Abbreviations used in this paper: EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; PFA, paraformaldehyde; PMN, polymorphonuclear cell; VE, vascular endothelial.
ECGS, 100

tumor necrosis factor (TNF)-

Mouse bEnd.3 endothelioma cells (Williams et al., 1989) were cultured as
telial cell growth supplement (Sigma Chemical Co., St. Louis, MO), 100 
then resuspended into M199, 20% FCS (for HUVEC) or DME, 10% FCS 
top microfuge for 5 min at 4

carried out on the tissue culture plates for 25 min on ice with occasional 

mli SDS-PAGE sample buffer (2% SDS, 10% glycerol, 65 mM Tris/HCl, 

against 

above). For detection of the human antigens monoclonal antibodies 
Transduction Laboratories, Lexington, KY), and against 

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated as de-
scribed (Warren, 1990), and cultured in M199, 20% FCS, 50 μg/ml endo-
thelial cell growth supplement (Sigma Chemical Co., St. Louis, MO), 100 μg/ml Heparin (Sigma Chemical Co.). Alternatively, HUVEC were pur-
chased Heparin (Sigma Chemical Co.), 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 10:1 or 2:1 ratio of leukocytes to 
EC as indicated. The cells were coincubated for 5 min at 37°C in a hu-
midified atmosphere. Nonadherent cells were washed off with PBS with-
out Ca²⁺ and Mg²⁺, 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 CaCl₂, 10 μg/ml leupeptin, 1 mM PMSF, 2 μg/ml 
pepsinat, 40 U/ml aprotinin, 30 μg/ml eglin C, or in boiling (95°C) Laem-
ml SDS-PAGE sample buffer (2% SDS, 10% glycerol, 65 mM Tris/HCl, 

recent work by Del Maschio et al. (1996) suggested that 

PMN adhesion to EC leads to the complete loss of β-cate-
inin and plakoglobin from total cell extracts, as well as to an at least partial disappearance of VE-cadherin and α-cate-
inin. 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 immu-
noblotting 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).

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 pres-
ence of protease inhibitors. Therefore, we changed the lysis conditions and extracted HUVEC and bound neutrophils with 
obiling (95°C) SDS-PAGE sample buffer. Subse-
potent 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 oc-
cur before cells are lysed and depends on the lysis condi-
tions.

To rule out a potential contribution of neutrophil pro-

tases in the degradation of the catenins Del Maschio et al. 
(1996) separately extracted HUVEC and PMNs with Tri-

ton X-100, pelleted the insoluble fraction, and then coin-
cubated 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 
minute, we analyzed whether the suspected proteolytic activity 
in the PMN lysates would be unstable. We prepared Tri-
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. 1B, 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. 1B, 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. 1B, lane 4). The two lysates were incubated for another 20 min on ice, and then analyzed by immunoblotting. As shown in Fig. 1B, 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 described 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 Triton X-100 instead of SDS. 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 (Fig. 2B, lane 4). Third, PMN lysates were added to the HUVEC lysates (lane 7). The two lysates were coincubated for another 20 min on ice, and then analyzed by immunoblotting. 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 (Fig. 2B, lane 4). The total lysates were incubated for 20 min on ice, and then analyzed by immunoblotting. 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 (Fig. 2B, lane 4).

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 were 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 (Fig. 2B, lane 4). The total lysates were incubated for 20 min on ice, and then analyzed by immunoblotting. 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 (Fig. 2B, lane 4).
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 TNF-α-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 non-denaturing 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 api-
cal 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 en-
dotheilal 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 neutro-
phil proteolytic activity. Alternatively, one would have to
find means to manipulate such a signaling pathway inde-
pendently of neutrophil adhesion.

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References
Aberle, H., H. Schwartz, and R. Kemler. 1996. Cadherin-catenin complex: pro-
tein interactions and their implications for cadherin function. J. Cell Bio-
chem. 61:514–523.
Allport, J.R., H. Ding, T. Collins, M.E. Gerritsen, and F.W. Luscinskas. 1997.
Endothelial-dependent mechanisms regulate leukocyte transmigration: a
process involving the proteasome and disruption of the VE-cadherin com-
p lex at endothelial cell-to-cell junctions. J. Exp. Med. 186:517–527.
Breier, G., F. Breierio, L. Caveda, R. Berthier, H. Schnurch, U. Gotsch, D.
Vestweber, W. Risau, and E. Dejana. 1996. Molecular cloning and expres-
sion of murine vascular endothelial-cadherin in early stage development of
cardiovascular system. Blood. 87:630–641.
Carlos, T.M., and J.M. Harlan. 1994. Leukocyte-endothelial adhesion mole-
cules. Blood. 84:2068–2101.
Dejana, E., M. Corada, and M. G. Lampugnani. 1995. Endothelial cell-to-cell
junctions. FASEB (Fed. Am. Soc. Eur. Biol.) J. 9:910–918.
Del Maschio, A., A. Zanetti, M. Corada, Y. Rival, L. Ruco, M.G. Lampugnani,
and E. Dejana. 1996. Polymorphonuclear leukocyte adhesion triggers the
disorganization of endothelial cell-to-cell adherens junctions. J. Cell Biol.
135:497–510.
Gotsch, U., E. Borges, R. Bosse, E. Böggeymeyer, M. Simon, H. Mossmann,
and D. Vestweber. 1997. VE-cadherin antibody accelerates neutrophil recruit-
ment in vivo. J. Cell Sci. 110:583–588.
Hahne, M., U. Jäger, S. Isenmann, R. Hallmann, and D. Vestweber. 1993. Five
TNF-inducible cell adhesion mechanisms on the surface of mouse endothe-
lium cells mediate the binding of leukocytes. J. Cell Biol. 121:655–664.
Huang, A., J.E. Manning, T.M. Bandak, M.C. Ratau, K.R. Hanser, and S.C. Sil-
verstein. 1993. Endothelial cell cytosolic free calcium regulates neutrophil
migration across monolayers of endothelial cells. J. Cell Biol. 120:1371–1380.
Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions
and regulation of cell adhesion. Trends Genet. 9:317–321.
Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the
head of bacteriophage T4. Nature. 227:680–685.
Lampugnani, M.G., M. Resnati, M. Raiteri, R. Pigott, A. Piscacane, G. Houen,
L.P. Ruco, and E. Dejana. 1992. A novel endothelial-specific membrane pro-
tein is a marker of cell–cell contacts. J. Cell Biol. 118:1511–1522.
Liaw, C.W., C. Cannon, M.D. Power, P.K. Kiboneka, and L.L. Rubin. 1990.
Identification and cloning of two species of cadherins in bovine endo-
thelial cells. EMBO (Eur. Mol. Biol. Organ.) J. 9:2701–2708.
Navarro, P., L. Caveda, F. Breierio, I. Mandoteanu, M. G. Lampugnani, and
E. Dejana. 1995. Catenin-dependent and -independent functions of vascular
endothelial cadherin. J. Biol. Chem. 270:30965–30970.
Rubin, L.L. 1992. Endothelial cells: adhesion and tight junctions. Curr. Opin.
Cell Biol. 4:830–833.
Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte
emigration: the multistep paradigm. Cell. 76:301–314.
Suzuki, S., K. Sano, and H. Tanihara. 1991. Diversity of the cadherin family: ev-
dence for eight new cadherins in nervous tissue. Cell Regul. 2:261–270.
Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regula-
tor. Science. 251:1451–1455.
Warren, J.B. 1990. Large vessel endothelial isolation. In The Endothelium: An
Introduction to Current Research. J.B. Warren, editor. Wiley-Liss, Inc., New
York. 263–272.
Williams, R.L., W. Risau, H.G. Zerwes, H. Drexler, A. Aguzzi, and E.F. Wag-
nar. 1989. Endothelioma cells expressing the polyoma middle T oncogene in-
duce hemangiomas by host cell recruitment. Cell. 57:1053–1063.
Zöllner, O., and D. Vestweber. 1996. The E-selectin ligand-1 is selectively acti-
vated in Chinese hamster ovary cells by the (1,3)-fucosyltransferases IV
and VII. J. Biol. Chem. 271:33002–33008.