

# Polymorphonuclear Leukocyte Adhesion Triggers the Disorganization of Endothelial Cell-to-Cell Adherens Junctions

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**Abstract.** Polymorphonuclear leukocytes (PMN) infiltration into tissues is frequently accompanied by increase in vascular permeability. This suggests that PMN adhesion and transmigration could trigger modifications in the architecture of endothelial cell-to-cell junctions. In the present paper, using indirect immunofluorescence, we found that PMN adhesion to tumor necrosis factor-activated endothelial cells (EC) induced the disappearance from endothelial cell-to-cell contacts of adherens junction (AJ) components: vascular endothelial (VE)-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin. Immunoprecipitation and Western blot analysis of the VE-cadherin/catenin complex showed that the amount of  $\beta$ -catenin and plakoglobin was markedly reduced from the complex and from total cell extracts. In contrast, VE-cadherin and  $\alpha$ -catenin were only partially affected. Disorganization of endothelial AJ by PMN was not accompanied by EC retraction or injury and was specific for VE-cadherin/catenin complex, since platelet/endothelial cell adhesion molecule 1 (PECAM-1) distribution at cellular contacts was unchanged. PMN adhesion to EC seems to be a prerequisite for VE-cadherin/catenin complex disorganization.

This phenomenon could be fully inhibited by blocking PMN adhesion with an anti-integrin  $\beta_2$  mAb, while it could be reproduced by any condition that induced increase of PMN adhesion, such as addition of PMA or an anti- $\beta_2$ -activating mAb. The effect on endothelial AJ was specific for PMN since adherent activated lymphocytes did not induce similar changes. High concentrations of protease inhibitors and oxygen metabolite scavengers were unable to prevent AJ disorganization mediated by PMN. PMN adhesion to EC was accompanied by increase in EC permeability in vitro. This effect was dependent on PMN adhesion, was not mediated by proteases and oxygen-reactive metabolites, and could be reproduced by EC treatment with EGTA. Finally, immunohistochemical analysis showed that VE-cadherin distribution was affected by PMN adhesion to the vessel wall in vivo too.

This work suggests that PMN adhesion could trigger intracellular signals in EC that possibly regulate VE-cadherin/catenin complex disorganization. This effect could increase EC permeability and facilitate PMN transmigration during the acute inflammatory reaction.

**E**NDOTHELIUM controls the passage of solutes and circulating cells from blood to tissues. This function requires highly effective intracellular and paracellular transport systems (Franke et al., 1988; Simionescu and Simionescu, 1991; Rippe and Haraldsson, 1994; Dejana et al., 1995). The paracellular permeability is controlled in large part by intercellular junctions. Endothelial cells (EC)<sup>1</sup> express at least four types of junctions: adher-

ence junctions (AJ), tight junctions, gap junctions, and complexus adherentes (Franke et al., 1988; Rubin, 1992; Dejana et al., 1995). Tight junctions and gap junctions are well developed in vessels where the control of permeability needs to be high, such as in large vessels, arterioles, or the brain microcirculation (Risau and Wolburg, 1990; Simionescu and Simionescu, 1991). Complexus adherentes is mostly developed in the lymphatic vasculature (Schmelz and Franke, 1993). In contrast, AJ are essentially ubiquitous. In postcapillary venules, where interchanges between blood and tissues need to be dynamic, paracellular permeability could be controlled essentially by AJ and by other adhesive molecules apparently not organized in specific junctional organelles, such as platelet/endothelial cell adhesion molecule 1 (PECAM-1) (Newman et al., 1990; DeLisser et al., 1994). AJ are formed by transmembrane  $\text{Ca}^{2+}$ -dependent adhesive proteins called cadherins, which

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1. *Abbreviations used in this paper:* AJ, adherens junction; EC, endothelial cell; ECL, enhanced chemiluminescence; ICAM, intercellular adhesion molecule; PECAM-1, platelet/endothelial cell adhesion molecule 1; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase; TNF, tumor necrosis factor; TX-100, Triton X-100; VE, vascular endothelial.

are associated inside the cells with a complex network of cytoskeletal molecules that in turn promote the anchorage to actin microfilaments (Takeichi, 1991; Geiger and Ayalon, 1992; Tsukita et al., 1992; Kemler, 1993; Klymkowsky and Parr, 1995).

An endothelial-specific cadherin has been identified: vascular endothelial (VE)-cadherin (or cadherin 5 or 7B4; Lampugnani et al., 1992; Breviario et al., 1995; Breier et al., 1996). VE-cadherin is complexed to cytoplasmic proteins named catenins, such as  $\alpha$ -catenin,  $\beta$ -catenin, plakoglobin, and p120<sup>cas</sup> (Lampugnani et al., 1995). EC also express significant amounts of N-cadherin. However, this molecule does not seem to play a major role in endothelial permeability since it is not organized at cell-to-cell contacts but remains diffused on the EC membrane (Salomon et al., 1992).

Polymorphonuclear leukocytes (PMN) are the first cells recruited from blood to sites of acute inflammatory reaction. Circulating PMN need first to adhere and then to cross the endothelial lining of postcapillary venules to enter the tissues. This process is a rapid event; once PMN stick to the luminal side of the endothelium, it takes only a few minutes to reach the subendothelial basal membrane (Marchesi and Florey, 1960). Adherent leukocytes can migrate toward endothelial cell-to-cell junctions and then transmigrate through them. This process is frequently accompanied by increase in vascular permeability and oedema formation (Larsen et al., 1980). Oedema is an essential part of the host defense system since it controls the supply of complement and other reactive substances from blood to an infected tissue to regulate local killing of microorganisms. Strong *in vivo* evidence indicates that PMN are required for oedema formation (Wedmore and Williams, 1981; Yi and Ulich, 1992), but the manner by which they could increase vascular permeability is still undetermined.

While a significant amount of information is available on the adhesive molecules regulating PMN adhesion to the vascular surface, we know little about the mechanisms that regulate the passage of PMN through endothelial junctions. An attractive hypothesis is that PMN adhesion could coordinate the opening of interendothelial junctions, facilitating in this way their transmigration. This process would also increase EC paracellular permeability and facilitate oedema formation. To test this possibility, in the present study we analyzed endothelial AJ organization upon PMN adhesion. We used human umbilical vein endothelium, which, like postcapillary venules, does not express organized tight junctions (Dejana et al., 1995).

We found that PMN adhesion to EC disrupted VE-cadherin/catenin complex. These molecules were lost from EC cell-to-cell contacts and a large amount of  $\beta$ -catenin and plakoglobin disappeared from VE-cadherin immunoprecipitates and total cell extracts. These findings suggest that PMN adhesion to EC could transfer intracellular signals that could induce AJ disorganization.

## Materials and Methods

### Materials

Reagents were purchased from the following sources: medium 199

(M199), RPMI 1640, and other culture reagents from Gibco-Europe (Paisley, UK); PBS (containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , unless otherwise specified) from Mascia Brunelli (Milano, Italy); tissue culture and flasks from Falcon (Becton Dickinson Labware, Lincoln Park, NJ); Lymphoprep from Nycomed Pharma AS (Oslo, Norway); Mowiol 4-88 from Calbiochem-Novabiochem Corp. (La Jolla, CA); Percoll and CNBr-activated Sepharose from Pharmacia (Uppsala, Sweden); paraformaldehyde, HRP, BSA, Tris, PMA, Ponceau S, human fibronectin, heparin, leupeptin, pepstatin, polyoxyethylenesorbitan monolaurate (Tween-20), 1,10-phenanthroline, PMSF, Triton X-100 (TX-100), DAB, EDTA, EGTA, FITC-labeled phalloidin, human elastase, and cathepsin G from Sigma Chemical Co. (St. Louis, MO); OCT compound from Miles Laboratories (Elkhart, IN); superoxide dismutase (SOD) and catalase from Boehringer Mannheim GmbH (Mannheim, Germany); human recombinant tumor necrosis factor (TNF) from Genzyme Corp. (Cambridge, MA); protein A-peroxidase conjugate and sulfo-nitrohydroxysuccinimido-biotin from Pierce Chemical Co. (Rockford, IL); NP-40 from BDH Laboratory Supplies (Latterworth, UK); eglin C from Ciba-Geigy (Basel, Switzerland); thimerosal from Merck (Darmstadt, Germany); aprotinin (Trasylol) from Bayer (Leverkusen, Germany); BB-94 (Batimastat) from British Biotechnology (Oxford, UK); all electrophoretic reagents from Bio Rad Laboratories (Richmond, CA);  $\text{Na}_2^{51}\text{CrO}_4$ , and enhanced chemiluminescence (ECL) from Amersham Intl. (Little Chalfont, UK); HRP-conjugated streptavidin from Biospa Division (Milano, Italy); TRITC-conjugated swine anti-rabbit IgG from Dakopatts (Glostrup, Denmark) and TRITC-conjugated goat anti-mouse, FITC-conjugated goat anti-mouse, and donkey anti-rabbit IgG from Jackson ImmunoResearch Laboratories Inc., (West Grove, PA); Transwell polycarbonate membrane inserts (0.4- $\mu\text{m}$  pore size) from Costar Corp. (Cambridge, MA).

### Antibodies

The following antibodies were used: mAb to human VE-cadherin TEA 1.31 (Leach et al., 1993) and mAb 7B4 (Lampugnani et al., 1992); mAb to human plakoglobin PG5.1 (Cowin et al., 1986; Franke et al., 1987), kindly donated by Prof. W. Franke (German Cancer Research Center, Heidelberg, Germany); rabbit purified Ig to human  $\alpha$ -catenin and  $\beta$ -catenin, kindly donated by Dr. D. Gulino (Laboratoire d'Hématologie, CEA, INSERM U217, Grenoble, France); mouse mAb to human PECAM-1 9G11 (British Biotechnology); blocking mAb to human integrin  $\beta_2$  TS1/18 (American Type Culture Collection, Rockville, MD); activating mAb to human integrin  $\beta_2$  KIM 127, kindly donated by Dr. Martyn Robinson (Celltech Therapeutics, Berkshire, UK); mouse mAb to E-selectin BBIG-E4 (R & D Systems Europe, Abingdon, UK).

### Leukocytes

Venous blood from healthy donors, who had not received any medication for at least 2 wk, was anticoagulated with trisodium citrate (3.8%, 1:9 vol/vol). PMN were isolated by dextran sedimentation followed by Lymphoprep gradient and hypotonic lysis of erythrocytes, as previously described (Del Maschio et al., 1989). PMN were washed and resuspended at the final concentration of  $3 \times 10^6$  per ml in M199 containing 20% heat-inactivated FBS. In some experiments, supernatants of activated PMN and PMN-derived membrane fragments were exposed to EC monolayers (for 10 min) to test their effect on the distribution of AJ components at cell-to-cell contacts.

Supernatants of activated PMN were obtained after exposure of  $3 \times 10^6$  PMN per ml to 100 nM PMA (10 min, 37°C). Cells were rapidly centrifuged, and supernatants were collected and kept at 4°C until use. Rough membrane fragments were recovered from the pellet of PMA-activated PMN (see above), resuspended in M199 (with 20% FBS), sonicated at 4°C by using 5-s bursts for 1 min in a Branson B15 Sonifier (Italscientifica, Milano, Italy), and kept at 4°C until use.

Mononuclear cells obtained from buffy coats of blood donations were centrifuged on Lymphoprep gradient, and then depleted of monocytes and natural killer cells by separation on 46% and subsequent density gradients of 50% Percoll (Allavena et al., 1991).

### Endothelial Cells

EC were isolated from human umbilical vein, cultured in M199 supplemented with 20% newborn calf serum, 50  $\mu\text{g}/\text{ml}$  endothelial cell growth supplement, and 100  $\mu\text{g}/\text{ml}$  heparin (M199 complete medium), and kept at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere, as previously described (Lampugnani et al., 1995).

## PMN Adhesion to EC

PMN were added to confluent EC monolayers treated with 100 U/ml TNF for 20 h, at a final physiological leukocyte-EC ratio of 10:1. In a series of experiments, PMN were layered on resting EC, and adhesion was stimulated by adding the activating anti- $\beta_2$  mAb KIM 127 (1–10  $\mu$ g/ml) or 10 nM PMA. PMN were therefore incubated for 5 min at 37°C (except otherwise specified), and nonadherent cells were eliminated by two washings with PBS. Cells were then fixed and permeabilized for immunofluorescence microscopy or extracted for immunoprecipitation and Western blot analysis, as described below.

In some experiments, before addition to TNF-treated EC, PMN were subjected to different treatments: 10 min (22°C) with the metalloprotease inhibitor BB-94 (1–2  $\mu$ g/ml); 20 min (22°C) with the anti- $\beta_2$  mAb TS1/18 (1:100 final dilution); 20 min (22°C) with a combination of SOD (300 U/ml) and catalase (300 U/ml); 30 min (22°C) with a mixture of serine protease inhibitors (100 U/ml aprotinin, 500  $\mu$ M leupeptin, and 30  $\mu$ g/ml eglin C). All of these agents remained in the medium during the adhesion assay. In some experiments, PMN were added to EC treated with the anti-E-selectin mAb BBIG-E4 (5  $\mu$ g/ml) for 1 h (37°C).

Preliminary experiments were performed with PMN resuspended in serum-free M199. This was, however, an unfavorable situation since EC monolayers were severely injured by adherent PMN. To preserve the endothelial integrity, we subsequently used PMN resuspended in M199 containing 20% heat-inactivated FBS. Under this condition, endothelial integrity was maintained during PMN adhesion. Indeed, endothelial toxicity, quantified as release of  $^{51}\text{Cr}$  from labeled EC (Westlin and Gimbrone, 1993), was 3.9% after 10 min of PMN adhesion, 3.5% after 10 min of cathepsin G exposure, and 3.2% in basal conditions.

## Immunofluorescence Microscopy

Immunofluorescence was analyzed as previously described in detail (Lampugnani et al., 1992). Cells were fixed with 3% paraformaldehyde and permeabilized with 0.5% TX-100. In some experiments, to confirm with other fixation methods the results obtained, cells were fixed either with methanol (5 min at  $-20^\circ\text{C}$ ) or fixed and permeabilized at the same time with 3% paraformaldehyde containing 0.5% TX-100 (3 min and an additional 15 min with paraformaldehyde alone; Ayalon et al., 1994). For PECAM-1 and F-actin staining, incubation with the primary PECAM-1 mAb was followed by the TRITC-conjugated goat anti-mouse secondary antibody, in the presence of FITC-labeled phalloidin (2 mg/ml). For double staining, the primary step was either with mouse anti-VE-cadherin in combination with rabbit anti- $\alpha$ -catenin or mouse anti-plakoglobin in combination with rabbit anti- $\beta$ -catenin. This was followed by TRITC-coupled goat anti-mouse in combination with FITC-coupled donkey anti-rabbit. Coverslips were then mounted in Mowiol 4-88 and examined with a microscope (Axioptot; Zeiss, Oberkochen, Germany). Images were recorded on films with a constant exposure of 40 s (T MAX P3200; Eastman Kodak Co., Rochester, NY).

## Immunoprecipitation and Western Blot Analysis

After PMN adhesion, confluent EC monolayers ( $\sim 2.4 \times 10^6$  cells) were washed twice with PBS at 4°C, and then extracted with 1 ml per petri dish of TBS containing: 2 mM  $\text{Ca}^{2+}$ , 15  $\mu$ g/ml leupeptin, 1 mM PMSF, 1 mg/ml pepstatin, 0.36 mM phenanthroline, 40 U/ml aprotinin, 30  $\mu$ g/ml eglin C, 1% TX-100, 1% NP-40, for 25 min on ice with occasional gentle agitation. Cell extracts were then centrifuged at 14,000 rpm for 5 min (4°C), and the supernatants was defined as a TX-soluble fraction. The monolayer was gently washed three times with TBS containing protease inhibitors, and then extracted with 0.5% SDS and 1% NP-40 in TBS containing protease inhibitors. The extract was collected, vigorously pipetted, and centrifuged at 14,000 rpm for 5 min (4°C). The supernatant was defined as a TX-insoluble fraction (Lampugnani et al., 1995).

In some experiments, PMN ( $2.1 \times 10^6$ /ml, corresponding to  $\sim 70\%$  of adherent cells) and TNF (100 U/ml)-treated EC ( $2.4 \times 10^6$ ) were extracted separately and subsequently mixed together for 20 min at 4°C. Samples were subsequently immunoprecipitated with 7B4-CNBr Sepharose and analyzed for the presence of VE-cadherin and catenins as described above.

For immunoprecipitation, cell extracts (TX-soluble fraction) were mixed with the anti-VE-cadherin 7B4 mAb covalently coupled to CNBr-activated Sepharose 4B (7B4-CNBr Sepharose). 7B4 was coupled at 1.8 mg per ml of CNBr-activated Sepharose 4B, according to the manufacturer's instructions. 7B4-CNBr Sepharose was then blocked and processed, ac-

cording to the manufacturer's instructions, and stored at 4°C in the presence of 0.02%  $\text{NaN}_3$ . Before use, 7B4-CNBr Sepharose was washed three times with TBS containing 1 mM PMSF, 2 mM  $\text{Ca}^{2+}$ , 40 U/ml aprotinin, and 1% TX-100. Cell extracts (1,000  $\mu$ l, corresponding to  $2.4 \times 10^6$  endothelial cells) were mixed with 7B4-CNBr Sepharose (10  $\mu$ l) for 2 h at 4°C under over-end rotation. Immunoprecipitates were washed six times with 500  $\mu$ l of 10 mM Tris, 0.5 M NaCl, 1 mM PMSF, 2 mM  $\text{Ca}^{2+}$ , 40 U/ml aprotinin, 0.05% NP-40, pH 7.4. Immunoprecipitates were treated with Laemmli sample buffer (Laemmli, 1970), containing 2-mercaptoethanol (5% final concentration), to elute bound proteins that were then resolved by SDS-PAGE. After SDS-PAGE, gels were incubated twice (15 min each) with transfer buffer (50 mM Tris-HCl, 95 mM glycine) and transferred electrophoretically to nitrocellulose filters (Towbin et al., 1979). After Ponceau S staining, filters were blocked with 10% lowfat milk in PBS, 0.05% Tween-20, and 0.01% thimerosal (washing buffer) and incubated with appropriate antibodies for 1 h at 22°C.

For immunoblotting, hybridoma culture supernatants containing mAbs directed to VE-cadherin and plakoglobin were used diluted 1:2 in washing buffer containing 1% BSA instead of milk, while rabbit IgG to  $\alpha$ -catenin and  $\beta$ -catenin were used at 30 and 200  $\mu$ g/ml, respectively, in the same buffer. Filters were washed, incubated (1 h at 22°C) with 2.5  $\mu$ g/ml rabbit anti-mouse IgG when required, and then incubated (1 h at 22°C) with protein A-peroxidase conjugate (1:10,000), followed by detection with ECL (Lampugnani et al., 1995).

In some experiments, after PMN adhesion, confluent EC monolayers were washed twice in serum-free M199 and twice in PBS; then the cells were treated with 0.5 mg/ml of sulfo-nitrohydroxysuccinimido-biotin in PBS for 20 min at 4°C. After the incubation, cells were washed twice with PBS and twice with serum-free medium, and then extracted (TX-soluble fraction), as described above. Labeled cell lysates were immunoprecipitated as previously described, and immunocomplexes were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose after blocking was incubated with streptavidin-peroxidase conjugate (1:5,000, final concentration) in PBS containing 1% BSA, 0.05% Tween-20, and 0.01% thimerosal for 1 h at 22°C, followed by detection with ECL.

## Immunohistochemistry

Fragments of human tonsils were obtained at surgery. Tissue fragments were embedded in OCT compound, snap frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until sectioning. Cryostat sections were fixed in acetone for 10 min at 22°C and immunostained with the anti-VE-cadherin mAb TEA 1.31 using avidin-biotin-peroxidase complex technique. The specimens were then processed and analyzed as previously described (Lampugnani et al., 1992).

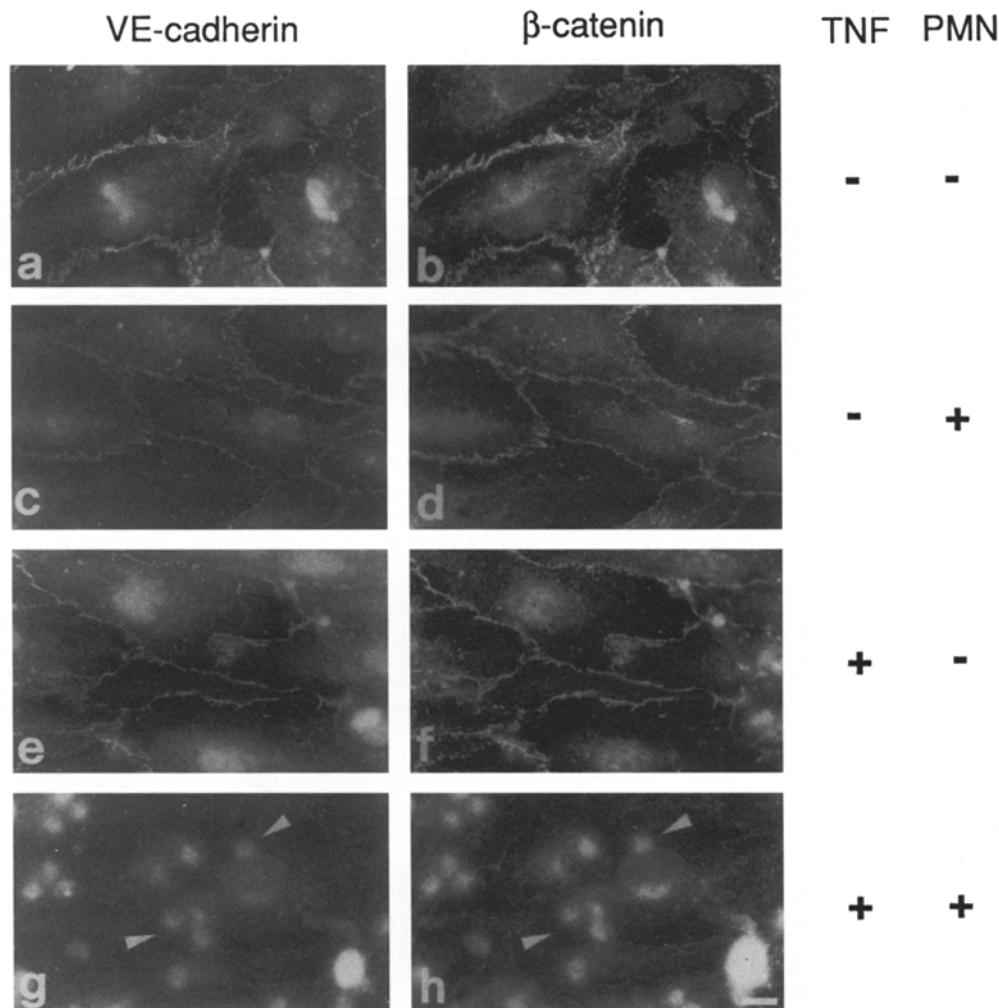
## Endothelial Permeability Assay

EC were cultured on fibronectin-coated (10  $\mu$ g/ml) polycarbonate membranes of Transwell inserts (0.4- $\mu$ m pore size) for 5 d (with daily refeeding), and then treated with 100 U/ml TNF for 20 h. Transwell inserts were subsequently filled with 100  $\mu$ l of M199 (+ 20% FBS) containing 6  $\mu$ g/ml of HRP or 100  $\mu$ l of PMN ( $3 \times 10^6$ /ml, resuspended in the same medium) either untreated or treated with TS1/18 mAb (1:100 final dilution, for 20 min) or SOD and catalase (300 U/ml, for 20 min), or a cocktail of protease inhibitors (100 U/ml aprotinin, 500  $\mu$ M leupeptin, 30  $\mu$ g/ml eglin C, for 30 min). In separate wells, EC were exposed to 2 mM EGTA. As previously described, in this assay, the passage of HRP reaches an equilibrium after 60 min of incubation (Navarro et al., 1995). After this interval, the medium in the lower compartment was collected and kept on ice until the assay of HRP enzymatic activity, as previously described (Ortiz de Montellano et al., 1988; Lampugnani et al., 1992; Navarro et al., 1995).

## Results

### Effect of PMN Adhesion on Endothelial AJ Organization

In confluent EC, AJ components are localized at cell-to-cell contacts. Indirect immunofluorescence analysis shows that antibodies directed to VE-cadherin and catenins ( $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin) give a fine, continuous staining of intercellular margins (Figs. 1, *a* and *b*, and 2, *a*



**Figure 1.** Effect of PMN adhesion on VE-cadherin and  $\beta$ -catenin localization in EC. Resting or TNF-activated EC (100 U/ml, 20 h), in the presence or absence of PMN ( $3 \times 10^6$ /ml), were double stained for VE-cadherin and  $\beta$ -catenin. PMN were incubated with EC for 5 min at 37°C. VE-cadherin and  $\beta$ -catenin were concentrated at cell-to-cell contacts in resting EC in the presence (c and d) or absence of PMN (a and b), or in TNF-activated EC in the absence of PMN (e and f). In contrast, in TNF-treated EC in the presence of PMN, VE-cadherin and  $\beta$ -catenin staining was markedly reduced (g and h). (Arrowheads) Adherent PMN. PMN adhesion was only observed on TNF-treated EC. Bar, 10  $\mu$ m.

and b). EC treatment with TNF (100 U/ml for 20 h) did not change VE-cadherin and catenin distribution (Figs. 1, e and f, and 2, e and f). When washed PMN ( $3 \times 10^6$ /ml) were added to resting EC monolayers, they adhered very poorly, and no significant change in the organization of endothelial AJ components was observed (Figs. 1, c and d, and 2, c and d). In contrast, when PMN adhered to TNF-treated EC monolayers (Figs. 1, g and h, and 2, g and h), VE-cadherin and catenin ( $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin) staining at interendothelial junctions was strongly reduced. This effect was rapid, partially detectable at 1 min after PMN adhesion and maximal within 5 min. No difference was observed by treating EC with TNF for 4, 6, or 20 h (not shown). Therefore, in all the experiments reported below, EC were treated with TNF, 100 U/ml, for a standard time of 20 h.

Maximal effect was observed when PMN were layered on TNF-treated EC at the concentration of  $3 \times 10^6$ /ml (experimental PMN/EC ratio of 10:1). Under this condition, about six to seven PMN were found adherent to one EC. When PMN were layered at a concentration of  $1.5 \times 10^6$ /ml (about three to four adherent PMN/EC), the effect was still observed, although with a minor intensity. In all the experiments reported below, a concentration of  $3 \times 10^6$  PMN per ml was used.

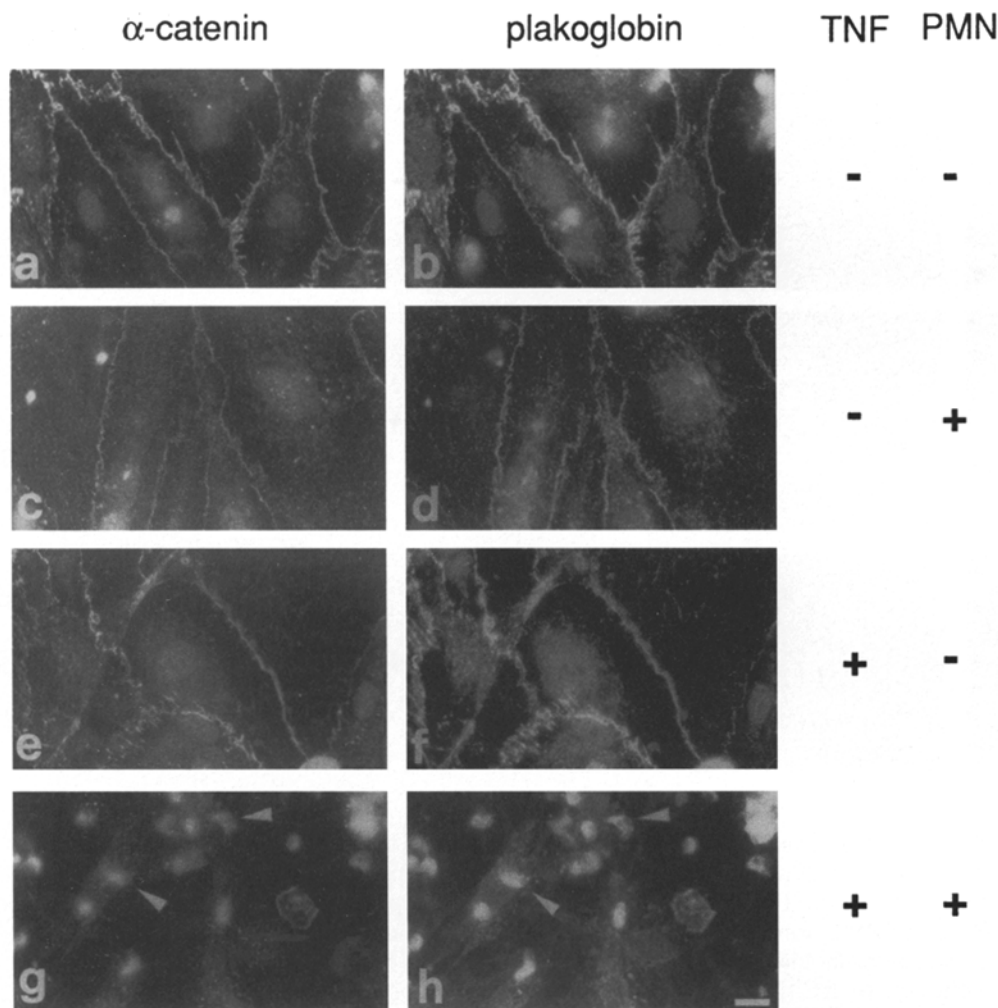
PECAM-1 and F-actin staining of EC shows that PMN adhesion did not induce EC retraction (Fig. 3). In addition, it also indicates that the effect was specific for VE-cadherin and catenins since PECAM-1 distribution remained unaffected.

Fig. 3 reports the results obtained with a 5-min incubation of PMN with EC, but comparable results were obtained by prolonging the incubation for up to 60 min. In parallel, no significant EC toxicity, as measured by  $^{51}\text{Cr}$  release, was observed up to 60 min of PMN adhesion to TNF-treated EC (not shown).

To explore the quantitative significance of the changes in junctional protein distribution upon PMN adhesion, cells were extracted and VE-cadherin immunoprecipitates blotted with antibodies to VE-cadherin and catenins, as previously described (Lampugnani et al., 1995).

In VE-cadherin immunoprecipitates, antibodies to  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin recognized bands of 100, 93, and 83–80 kD, respectively (Fig. 4). As previously described, plakoglobin appears in all the experiments as a triplet (Lampugnani et al., 1995).

Addition of PMN to TNF-treated EC induced a marked reduction of  $\beta$ -catenin and plakoglobin associated to VE-cadherin. VE-cadherin and  $\alpha$ -catenin appeared less affected (Fig. 4). A similar reduction of the components of



**Figure 2.** Effect of PMN adhesion on  $\alpha$ -catenin and plakoglobin localization in EC. Resting or TNF-activated EC (100 U/ml, 20 h), in the presence or absence of PMN ( $3 \times 10^6$ /ml), were double stained for  $\alpha$ -catenin and plakoglobin. PMN were incubated with EC for 5 min at 37°C.  $\alpha$ -Catenin and plakoglobin were concentrated at cell-to-cell contacts in resting EC in the presence (c and d) or absence of PMN (a and b), or in TNF-activated EC in the absence of PMN (e and f). In contrast, in TNF-treated EC in the presence of PMN,  $\alpha$ -catenin and plakoglobin staining was markedly reduced (g and h). (Arrowheads) Adherent PMN. PMN adhesion was only observed on TNF-treated EC. Bar, 10  $\mu$ m.

VE-cadherin/catenin complex was observed in the total cell extracts (Fig. 4).

When Western blot was performed on the TX-insoluble fraction of the total cell extracts (see Materials and Methods), a similar decrease in  $\beta$ -catenin and plakoglobin was observed (Fig. 5).

Western blot analysis of extracts of PMN alone showed that these cells did not express measurable levels of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin (not shown). Therefore, the AJ components detected in the assays are exclusively of endothelial origin.

Treatment of EC with TNF in the absence of PMN did not significantly change the amount of AJ components (Fig. 4). In a few experiments, we observed some decrease of VE-cadherin and catenins even when EC were exposed to PMN in the absence of TNF pretreatment (see, for instance, Fig. 4 A). In these experiments, however, some adhesion of PMN to resting EC was observed. We interpreted these results as a consequence of a slight activation of PMN during the isolation and washing procedure.

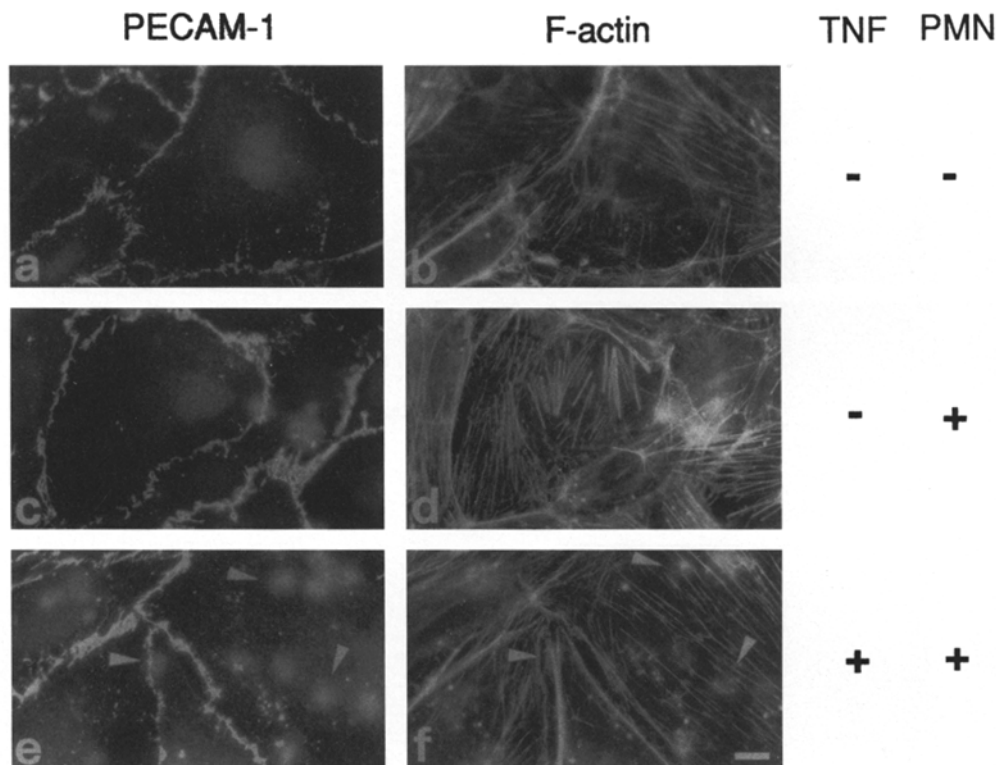
Finally, to evaluate the degree of VE-cadherin internalization, TNF-treated EC were incubated with PMN for 5 min. Cells were then exposed to sulfo-nitrohydroxysuccinimido-biotin and immunoprecipitated with a VE-cadherin mAb. By avidin-HRP blotting, we detected one band with

the expected apparent molecular mass of 130 kD. A decrease of  $\sim 30\%$  was observed in the amount of VE-cadherin immunoprecipitated with adherent PMN, with respect to the amount immunoprecipitated in the absence of PMN (not shown). These data indicate that, upon PMN adhesion, VE-cadherin's disappearance from cell-to-cell contacts is partially due to its internalization. However, a large part of the loss in immunofluorescence staining at intercellular junctions is likely related to the fact that the molecule diffuses on the cell surface.

#### **PMN Adhesion to EC Is a Prerequisite for AJ Disorganization**

To investigate whether the physical contact between intact PMN and EC is required for AJ disorganization, PMN and TNF-treated EC were lysed and extracted separately. The two cell extracts were then mixed together (see Materials and Methods), and the components of the VE-cadherin/catenin complex were analyzed by immunoprecipitation and Western blot. In these conditions, the amount of VE-cadherin and catenins remained unchanged (Fig. 4 B).

In other experiments, we studied whether the block of PMN adhesion would inhibit AJ disassembly in TNF-treated EC. As above, EC were treated with TNF for 20 h.



**Figure 3.** PECAM-1 and F-actin organization in EC in the presence of adherent PMN. Cells were double stained for PECAM-1 and F-actin in resting (*a-d*) or TNF-activated EC (100 U/ml, 20 h) (*e* and *f*), either in the presence (*c-f*) or absence (*a* and *b*) of PMN ( $3 \times 10^6$ /ml). PMN were incubated with EC for 5 min at 37°C. A PECAM-1 mAb gave an intense, continuous staining of EC at cell-to-cell contacts. Both PECAM-1 and F-actin distribution remained identical in all the situations considered. EC retraction or opening of gaps at cell-to-cell contacts could never be observed. (Arrowheads) Adherent PMN. Bar, 10  $\mu$ m.

In these conditions, EC expressed intercellular adhesion molecule (ICAM) 2, increased ICAM-1 and vascular cellular adhesion molecule (VCAM) 1 levels, but not significant E-selectin (Bevilacqua, 1993). Since PMN commonly do not bind VCAM-1,  $\beta_2$  integrin receptors should play a major role in promoting their adhesion to EC. Indeed, when PMN were treated with the anti- $\beta_2$  blocking mAb TS1/18 and then added to TNF-treated EC, their adhesion was essentially abolished. Basal and TNF-dependent adhesion was  $20 \pm 8$  and  $173 \pm 12 \times 10^3$  PMN per well, respectively, in the presence of an irrelevant control antibody, and became  $7 \pm 2$  and  $32 \pm 11 \times 10^3$  PMN per well, respectively, in the presence of TS1/18 mAb. As expected, PMN adhesion was unaffected by EC treatment with the E-selectin-blocking mAb BBIG-E4 (not shown).

As assessed by immunofluorescence, in the presence of PMN treated with TS 1/18, the distribution of VE-cadherin,  $\beta$ -catenin (Fig. 6),  $\alpha$ -catenin, and plakoglobin (not shown) in TNF-treated EC remained unchanged. Similarly, by immunoprecipitation/Western blot analysis of AJ components in the presence of TS 1/18 mAb, no significant change in VE-cadherin/catenin complex was observed (Fig. 7, lane 3).

We then asked whether TNF activation of EC was necessary for the observed changes in AJ organization. We therefore analyzed the effect of PMN adhesion on resting EC. Adhesion was first induced by exposing PMN to 10 nM PMA for 5 min. Plakoglobin (Fig. 8), VE-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin (not shown) distribution at EC junctions was not modified by PMA alone (Fig. 8, compare *a* with *b*). However, when PMN were activated by PMA, their adhesion was increased by fivefold, and AJ components disappeared at endothelial cell-to-cell contacts (Fig. 8, compare *c* with *a* and *b*).

PMN adhesion was then induced by the  $\beta_2$ -activating mAb KIM 127 (Ortlepp et al., 1995). When PMN were exposed to KIM 127 (10  $\mu$ g/ml) for 5 min, their adhesion to resting EC was increased by 10-fold (not shown). In these conditions, staining of  $\alpha$ -catenin,  $\beta$ -catenin, VE-cadherin (not shown), and plakoglobin was essentially undetectable (Fig. 8 *f*). When KIM 127 was used at a lower concentration (1  $\mu$ g/ml), PMN adhesion was increased on the EC monolayer but in a nonhomogeneous way. Interestingly, AJ components disappeared from cell-to-cell contacts only where PMN were firmly adherent to EC, but they remained intact in the areas devoid of adherent PMN (Fig. 8, compare *d* with *e*).

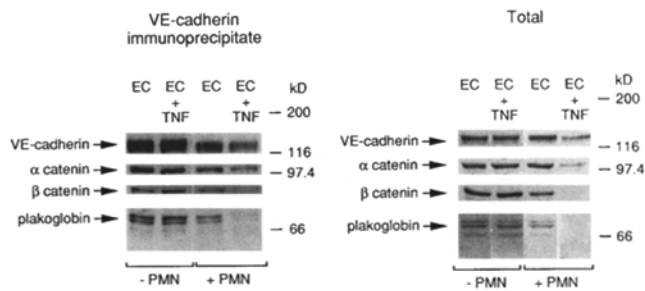
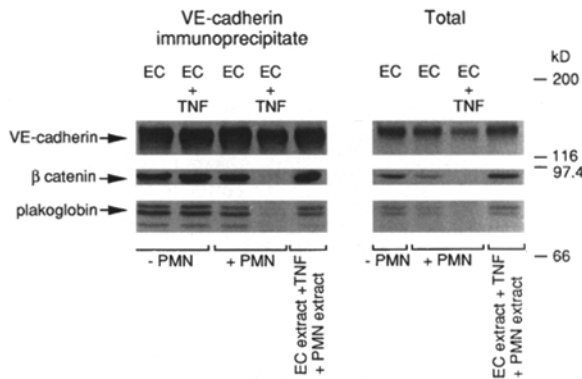
To test the specificity of the effect of PMN on endothelial AJ, we tested T-lymphocyte adhesion to EC. T-lymphocytes ( $3 \times 10^6$ /ml) were added to EC treated with TNF (100 U/ml, 20 h), and then stimulated with 10 nM PMA for 10 min. As shown in Fig. 9, endothelial AJ organization was not modified by adherent T-lymphocytes. In these conditions, the number of adherent T-lymphocytes was seven to eight per one EC. This value was comparable to that of adhering PMN (six to seven per one EC) in our standard conditions (see also above).

#### **PMN-derived Proteases and Toxic Oxygen Metabolites Did Not Modify Endothelial AJ**

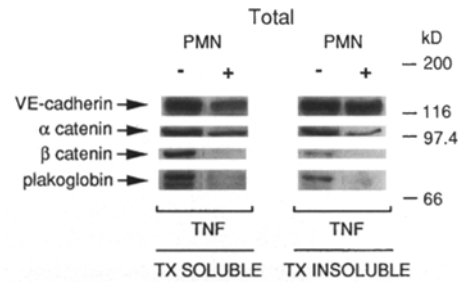
To further investigate the role of PMN adhesion versus that of PMN-released soluble products, we asked whether PMN-derived proteases or toxic oxygen metabolites could be responsible for the observed changes in VE-cadherin/catenin complex.

We first evaluated the effect of the combination of the whole products released by activated PMN. Supernatants



**A****B**

**Figure 4.** (A) Effect of PMN adhesion on VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin coimmunoprecipitation. (VE-cadherin immunoprecipitate) Confluent EC monolayers were treated either with TNF (100 U/ml) or medium alone for 20 h. PMN ( $3 \times 10^6$ /ml) or medium alone was added for 5 min at 37°C. EC were then washed and extracted as described (see Materials and Methods). Cell extracts were immunoprecipitated with 7B4-CNBr Sepharose, run in a 7% SDS-PAGE under reducing conditions, and then analyzed for the presence of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin by immunoblotting. To save antibody and to directly compare the level of VE-cadherin and catenins in the same sample, after blotting the nitrocellulose was cut perpendicularly to the direction of protein run, separating the areas of VE-cadherin and each catenin band as described (Lampugnani et al., 1995). This was done with the reference of molecular weight standards. The sheets were then reacted with the appropriate antibody. (Total) Aliquots of cell extracts were run in 7% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and then analyzed for the presence of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin by immunoblotting. (B) Effect of the physical contact between intact PMN and EC on VE-cadherin,  $\beta$ -catenin, and plakoglobin coimmunoprecipitation. (VE-cadherin immunoprecipitate) Confluent EC monolayers were treated either with TNF (100 U/ml) or medium alone for 20h. PMN ( $3 \times 10^6$ /ml) or medium alone was added for 5 min at 37°C, and cells were washed and extracted. EC and PMN were also extracted separately, and then mixed together as described in Materials and Methods. Cell extracts were immunoprecipitated with 7B4-CNBr Sepharose, run in a 7% SDS-PAGE under reducing conditions, and then analyzed for the presence of VE-cadherin,  $\beta$ -catenin, and plakoglobin by immunoblotting as described for A. (Total) As for A. The migration of molecular mass markers is shown on the right.



**Figure 5.** Western blots of Triton-soluble and -insoluble fractions of whole EC lysates. Confluent EC monolayers were treated either with TNF (100 U/ml) or medium alone for 20 h. PMN ( $3 \times 10^6$ /ml) or medium alone was added for 5 min at 37°C, and cells were washed and extracted as described in Materials and Methods section. Aliquots of cell extracts were run in 7% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and then analyzed for the presence of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin by immunoblotting.

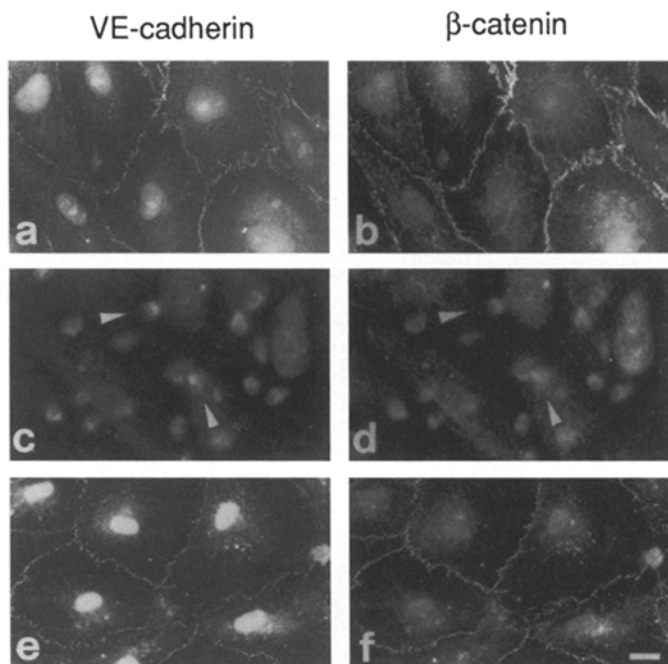
of PMA-activated PMN, added to EC for 10 min, did not modify AJ components as assessed by immunofluorescence (not shown) and by immunoprecipitation and Western blot analysis (Fig. 7).

To substantiate the effect of unstable components released during PMN activation, PMN were layered on Transwell filters (0.4- $\mu$ m pore size) to hinder their direct contact with EC monolayers cultured in the lower compartment. PMN were then exposed to PMA, and VE-cadherin/catenin distribution was examined by indirect immunofluorescence. After 10 min of PMN exposure to PMA (0.1 to 1,000 nM), AJ organization was unchanged (not shown).

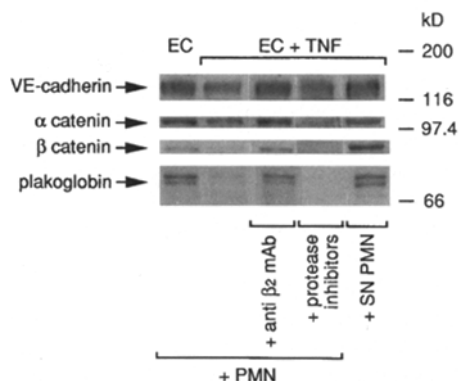
Release of proteolytic enzymes, such as elastase and cathepsin G, by activated PMN may induce endothelial damage (Peterson, 1989; Lampugnani et al., 1992). For this reason, in this work, adhesion assays were performed in the presence of 20% heat-inactivated FBS, which contains a large amount of natural protease inhibitors (Travis and Salvesen, 1983). For an additional block of PMN proteases, the effect of PMN adhesion to TNF-activated EC was tested in the presence of a cocktail of inhibitors (100 U/ml aprotinin, 500  $\mu$ M leupeptin, and 30  $\mu$ g/ml eglin C). The antiprotease cocktail added during the adhesion assay did not prevent the disappearance of AJ components, as assessed by immunofluorescence (Fig. 10, e and f) and immunoprecipitation–Western blot analysis (Fig. 7, lane 4).

We next investigated the direct effect of a combination of elastase and cathepsin G directly added to EC cultured medium. Endothelial VE-cadherin/catenin complex organization was not altered upon EC exposure to a high concentration (1  $\mu$ g/ml) of both elastase and cathepsin G for 10 min (Fig. 10, i and j). During PMN activation, part of elastase and cathepsin G activity might remain associated to the PMN membrane (Owen et al., 1995). However, when membrane fragments derived from PMA-activated PMN (see Materials and Methods) were incubated on EC monolayers for 10 min, no detectable modification of VE-cadherin and catenin immunofluorescence staining was observed (not shown).

Leukocyte metalloproteases released during inflammatory diseases play a role in oedema formation and leuko-



hesis and VE-cadherin and  $\beta$ -catenin disappearance (compare *e* and *f* with *a* and *b*). Comparable results were obtained when EC were double stained with antibodies to  $\alpha$ -catenin and plakoglobin. (Arrowheads) Adherent PMN. Bar, 10  $\mu$ m.



**Figure 7.** Effect of the anti- $\beta_2$  integrin blocking mAb, protease inhibitors, and supernatant from PMA-activated PMN on VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin coimmunoprecipitation. Confluent EC monolayers were treated either with TNF (100 U/ml) or medium alone for 20 h. PMN ( $3 \times 10^6$ /ml) were previously treated with anti- $\beta_2$  mAb (1:100 final concentration for 20 min at 22°C) or with a cocktail of protease inhibitors (100 U/ml aprotinin, 500  $\mu$ M leupeptin, and 30  $\mu$ g/ml eglin C) for 20 min at 22°C. The agents were then kept in the medium during PMN adhesion assay that was for 5 min at 37°C, as previously described. PMN supernatants were produced by treating PMN with 100 nM PMA (10 min at 37°C). PMN were then rapidly centrifuged at 14,000 rpm, and the supernatant was incubated with TNF-treated EC for 10 min. At the end of the incubation period, cells were washed and extracted. Cell extracts were then analyzed by immunoprecipitation with 7B4-CNBr Sepharose and immunoblotting of VE-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin as described in the legend to Fig. 4. Molecular mass markers are shown on the right.

TNF	PMN	+ anti $\beta_2$
+	-	-
+	+	-
+	+	+

**Figure 6.** PMN treatment with the anti- $\beta_2$  integrin blocking mAb TS1/18 prevents the disappearance of VE-cadherin and  $\beta$ -catenin staining at cell-to-cell contacts. TNF-activated EC (100 U/ml, 20 h) in the presence or absence of PMN ( $3 \times 10^6$ /ml) were double stained for VE-cadherin and  $\beta$ -catenin. PMN either untreated or treated with TS1/18 (1:100 final dilution; 20 min at 22°C) were incubated with EC for 5 min at 37°C. VE-cadherin and  $\beta$ -catenin staining was concentrated in the regions of cell-to-cell contact in the absence of PMN (*a* and *b*). As previously shown, in the presence of PMN, staining of VE-cadherin and  $\beta$ -catenin disappeared from cell-to-cell contacts (*c* and *d*). Addition of TS1/18 blocked PMN ad-

cyte infiltration (Goetzl et al., 1996). We have therefore performed experiments with PMN treated with the metalloprotease inhibitor BB-94 (Botos et al., 1996). As assessed by immunofluorescence analysis, BB-94 (1–2  $\mu$ g/ml) did not prevent the disruption of endothelial AJ components after PMN adhesion to TNF-treated EC (not shown).

Superoxide anions and hydrogen peroxide that originated during PMN respiratory burst could affect EC monolayer integrity by inducing cell retraction and increasing endothelial permeability (Sacks et al., 1978; Carlos and Harlan, 1994; Bradley et al., 1995). We therefore tested whether the generation of these toxic oxygen metabolites could be responsible for the changes in endothelial AJ organization observed. Immunofluorescence analysis showed that VE-cadherin/catenin complex of TNF-treated EC junctions was similarly disorganized by adherent PMN, either untreated or treated with 300 U/ml of SOD and catalase (Fig. 10, *g* and *h*).

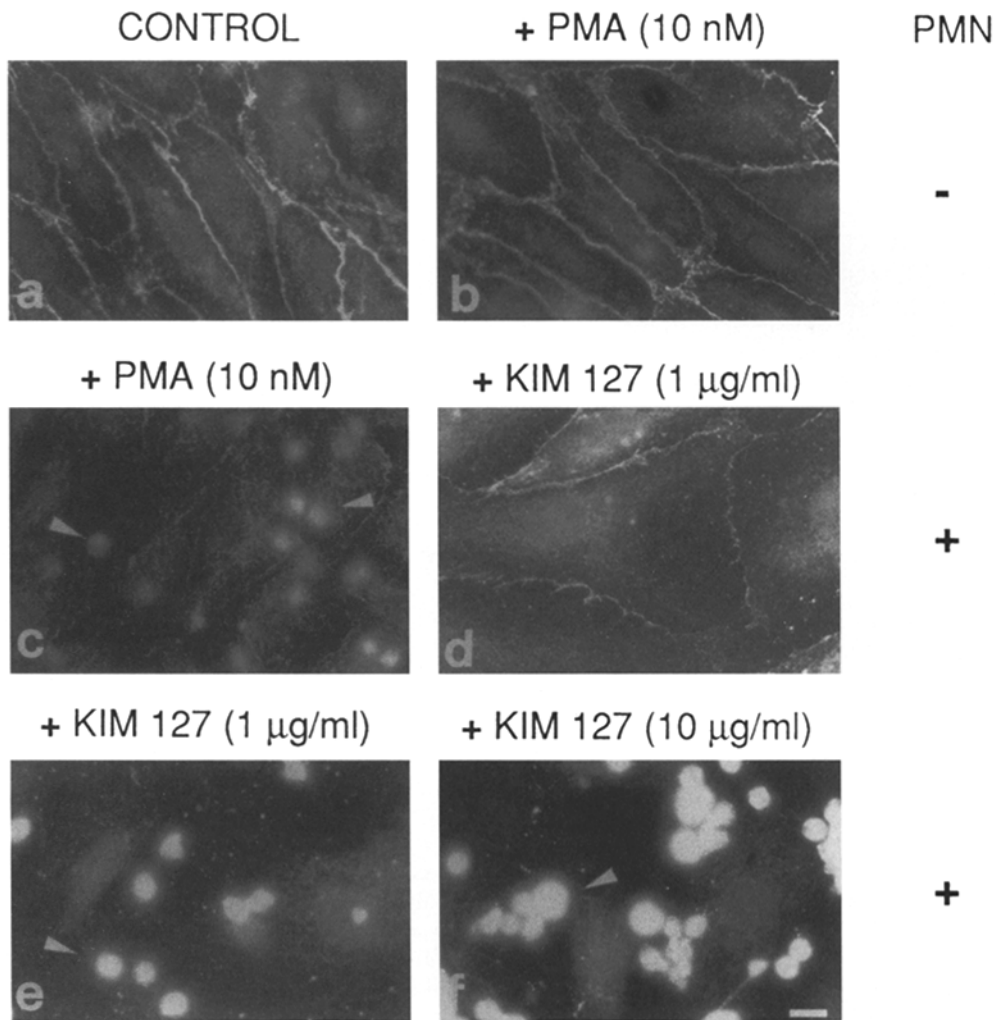
In all of the experiments above, the concentration of inhibitors selected was highly in excess with respect to that required to block the total amount of proteases and oxygen metabolites potentially released by an equal number of activated PMN (Sacks et al., 1978; Weiss et al., 1981; Owen et al., 1995).

Taken together, these results strongly suggest that the changes in endothelial AJ organization induced by PMN are not mediated by the extracellular release of soluble reactive components.

#### AJ Disorganization Induced by PMN Adhesion Is Accompanied by an Increase in Endothelial Permeability

We then studied whether the observed changes in VE-cad-





**Figure 8.** Effect of PMA or KIM 127-induced PMN adhesion on AJ organization in EC. Resting EC were stained for plakoglobin in conditions in which PMN adhesion was induced by PMA (10 nM) or KIM 127 (1 µg/ml, 10 µg/ml) for 5 min at 37°C. In the absence of PMN, plakoglobin staining was concentrated in the regions of cell-to-cell contact both in resting (a) and PMA-activated EC (b). In the presence of PMN, when adhesion was induced by PMA (c) or KIM 127 (e and f), plakoglobin staining disappeared at endothelial AJ. Adherent PMN were not homogeneously distributed in the endothelial monolayer after their exposure to 1 µg/ml KIM 127. (d and e) Two different areas from the same coverslip, plakoglobin staining disappeared only in the area with adherent PMN (compare d with e). Comparable results were obtained when EC were stained with VE-cadherin,  $\alpha$ -catenin, or  $\beta$ -catenin antibodies. (Arrowheads) Adherent PMN. Bar, 10 µm.

herin and catenins at EC contacts could have biological consequences. It was previously observed that the disorganization of VE-cadherin/catenin complex by EGTA increases EC permeability (Lampugnani et al., 1992). The effect of EGTA presents many similarities with that of PMN adhesion. It is rapid (maximal effect within 5–10 min) and leads to a complete disappearance of VE-cadherin and catenins at EC contacts (Lampugnani et al., 1992; Ayalon et al., 1994; Dejana et al., 1995).

As shown in Fig. 11, PMN adhesion to TNF-treated EC increased HRP permeability in a way comparable to that induced by 2 mM EGTA. TNF treatment alone or addition of PMN to resting EC did not change this parameter. Consistent with the effect on endothelial AJ disorganization, PMN treatment with the anti- $\beta_2$  blocking mAb TS1/18, treatment with either the cocktail of protease inhibitors (100 U/ml aprotinin, 500 µM leupeptin, and 30 µg/ml eglin C), or the combination of 300 U/ml of both SOD and catalase was ineffective.

Finally, since T-lymphocyte adhesion to EC did not change AJ organization, we investigated whether addition of T-lymphocytes could affect EC monolayer permeability.

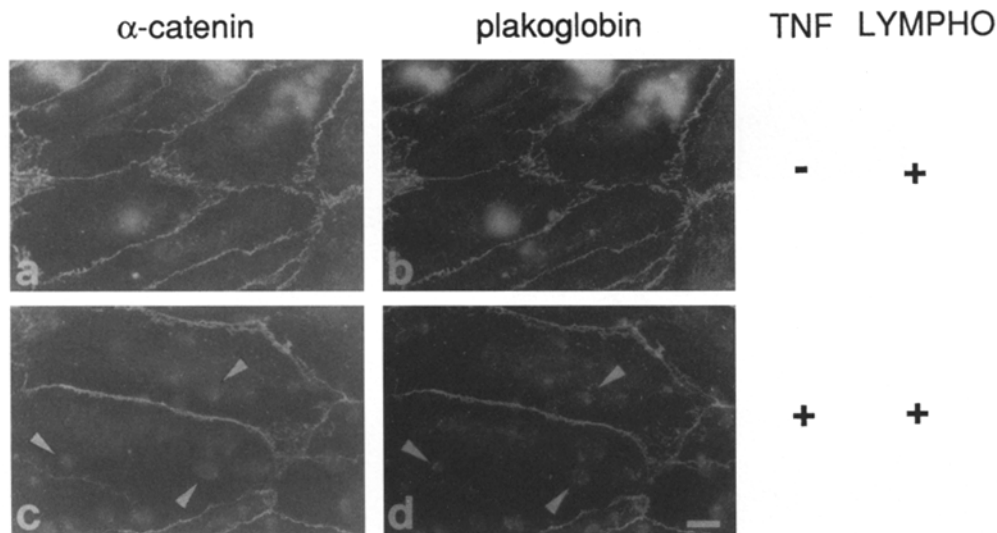
In these experiments, EC were treated with TNF and T-lymphocytes were treated with PMA as described for

Fig. 9. Permeability was measured as described above for PMN adhesion to EC. Adhesion of T-lymphocytes induced only a 33–40% increase in permeability (range of two experiments) with respect to the permeability of control EC treated with TNF and PMA in the absence of T-lymphocytes. This value was ~10 times less than the percentage increase in permeability induced by an equal number of adhering PMN.

These data suggest that the dissociation of VE-cadherin and catenin complex induced by PMN decreases the capacity of EC to limit the passage of soluble high molecular weight molecules through the junctions.

#### **PMN Adhesion to EC In Vivo Changes VE-cadherin Distribution**

We investigated VE-cadherin localization in tissue sections of five cases of chronic recurrent tonsillitis by immunohistochemistry. In postcapillary high endothelial venules without trafficking cells, immunostaining for VE-cadherin was localized at endothelial cell-to-cell contacts (Fig. 12 a). High endothelial venules with adherent PMN showed a reduction of VE-cadherin expression that was no longer detectable at the intercellular borders (Fig. 12 b).



**Figure 9.** Effect of T-lymphocyte (LYMPHO) adhesion on  $\alpha$ -catenin and plakoglobin localization at EC junctions. EC activated with TNF (100 U/ml, 20 h; c and d) or an equal amount of buffer (a and b) were double stained for  $\alpha$ -catenin and plakoglobin. Either resting or PMA (10 nM)-activated T-lymphocytes ( $3 \times 10^6$ /ml) were added to EC for 10 min at 37°C.  $\alpha$ -Catenin and plakoglobin staining was concentrated in the regions of cell-to-cell contact either in the absence (a and b) or presence (c and d) of lymphocyte adhesion. (Arrowheads) Adherent T-lymphocytes. Comparable data were obtained when EC were double stained with VE-cadherin and  $\beta$ -catenin. Bar, 10  $\mu$ m.

## Discussion

PMN adhesion to the vascular lining accompanies the first stages of the acute inflammatory reaction. In response to chemokines and to the expression of EC adhesive molecules, PMN first roll on the endothelial surface. This stage is then followed by a more firm adhesion, and then by a rapid transmigration of PMN through EC intercellular junctions (Carlos and Harlan, 1994; Springer, 1994; Imhof and Dunon, 1995). Adhesion is required for PMN extravasation, but the mechanisms that regulate the opening of EC junctions and the passage of PMN are still obscure.

PMN interaction with EC *in vivo* also controls fluid efflux through the blood vessel wall leading to tissue oedema (Wedmore and Williams, 1981; Yi and Ulich, 1992). While the role of PMN in this process was clearly determined, the manner in which these cells increase vessel permeability remains obscure.

In this study, we found that PMN adhesion to EC monolayers induces endothelial AJ disassembly. Upon adhesion of PMN, VE-cadherin and associated catenins ( $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin) disappeared from endothelial AJ. This effect was rapid, maximal within 5 min, and not related to EC retraction or toxicity. F-actin and PECAM-1 staining of EC showed that, within the time frame of the experiments, EC monolayers remained intact and the PECAM-1 amount and distribution was essentially unaffected.

Immunoprecipitation and Western blot analysis of AJ components showed that, while the amount of VE-cadherin and  $\alpha$ -catenin was only partially decreased,  $\beta$ -catenin and plakoglobin were markedly reduced.

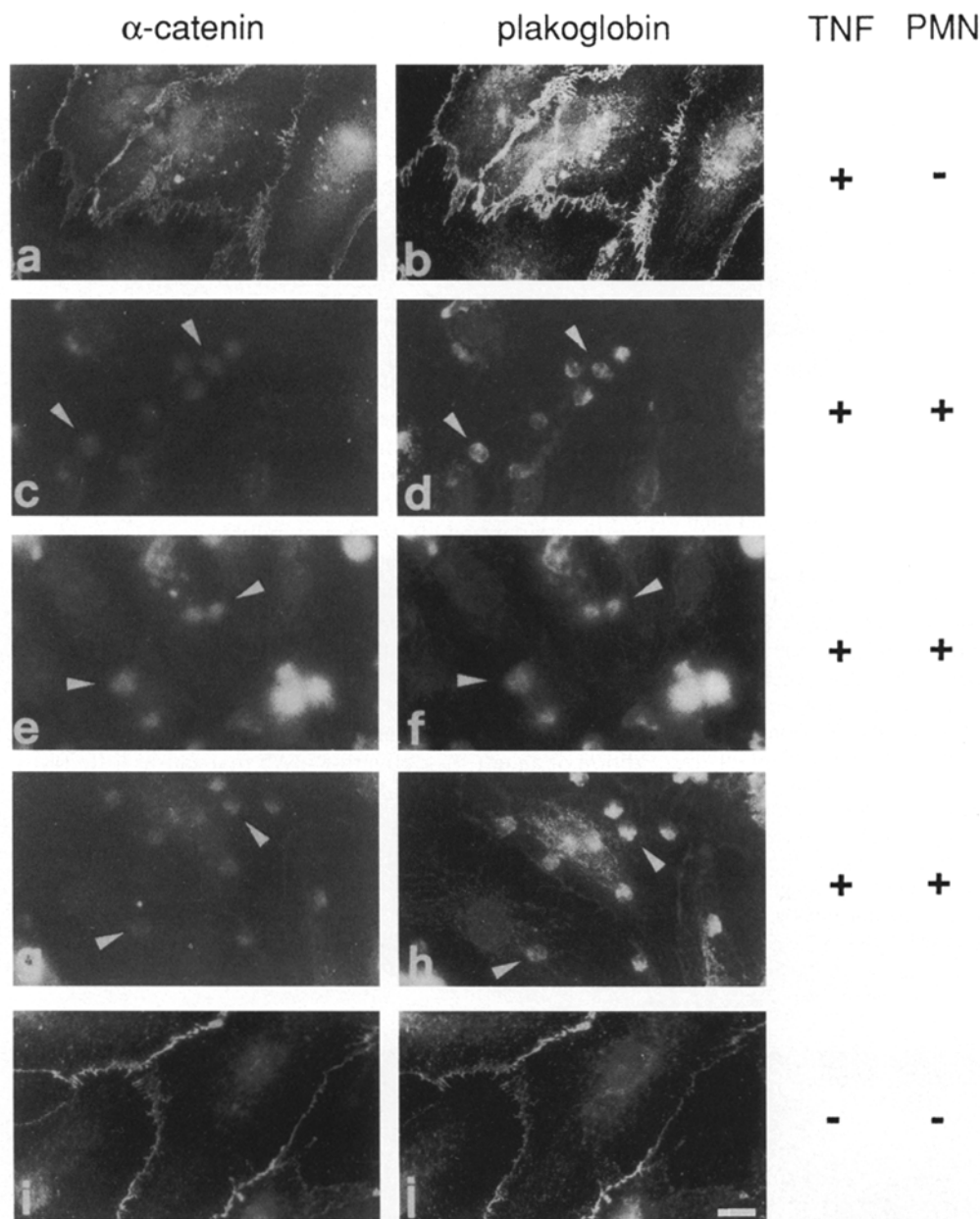
$\alpha$ -Catenin does not directly bind to VE-cadherin, but it associates to the complex via its binding to  $\beta$ -catenin and plakoglobin (Aberle et al., 1994; Hülken et al., 1994). Therefore, the total  $\alpha$ -catenin coimmunoprecipitated with VE-cadherin is the sum of the amount of  $\alpha$ -catenin associ-

ated to both  $\beta$ -catenin and plakoglobin. It is therefore conceivable that the total decrease in  $\alpha$ -catenin, observed after PMN adhesion to EC, is lower than that of the other two catenins.

The fact that VE-cadherin disappeared in immunofluorescence and was only partially decreased in immunoprecipitation might be seen as a discrepancy. Since only a small amount of VE-cadherin (<30%) was internalized (see Results), it is likely that the majority of the molecule simply diffuses on the cell surface after PMN adhesion. In other conditions, it was found that, when VE-cadherin is not organized and clustered at junctions, it is poorly detectable in immunofluorescence staining even if its amount on the cell surface is not reduced (see, for instance, Lampugnani et al., 1992, 1995; Dejana et al., 1995).

We still do not know the complete mechanism that leads to the disorganization of VE-cadherin/catenin complex induced by PMN; however, their firm adhesion to the EC surface appears to be a prerequisite. AJ were disrupted in all the conditions in which PMN adhesion was significantly increased, including activation of EC with TNF or activation of PMN with PMA or  $\beta_2$ -activating antibodies. In contrast, when PMN adhesion to TNF-treated EC was prevented by  $\beta_2$ -blocking antibodies or by separating PMN by a Transwell filter, AJ remained intact. Furthermore, in the same culture well, AJ were disorganized only in areas of firm PMN adhesion, and they remained unchanged where PMN were absent.

Through their adhesion, PMN could transfer intracellular signals to EC in different ways. One possibility is through the engagement of adhesive molecules. ICAM-1 is one of the major endothelial adhesive molecules for PMN in the conditions used in this study (i.e., long-lasting activation of EC with TNF, activation of PMN with PMA or anti- $\beta_2$  mAb; Bevilacqua, 1993; Carlos and Harlan, 1994), and indeed, the blockage of the receptors for ICAM-1, by  $\beta_2$  antibodies, induces inhibition of PMN adhesion and AJ



**Figure 10.** Disappearance of VE-cadherin and  $\beta$ -catenin from cell-to-cell contacts is not prevented by PMN treatment with antiprotease inhibitors or oxygen metabolite scavengers. EC were double stained for  $\alpha$ -catenin and plakoglobin. The staining of both catenins was concentrated at cell-to-cell contacts in TNF-activated (100 U/ml, 20 h) EC in the absence of PMN (*a* and *b*). Adhesion of resting PMN (5 min, 37°C) induced the disappearance of  $\alpha$ -catenin and plakoglobin staining at endothelial AJ (*c* and *d*). When PMN adhesion was performed in the presence of a cocktail of protease inhibitors (100 U/ml aprotinin, 500  $\mu$ M leupeptin, 30  $\mu$ g/ml eglin C; *e* and *f*) or of a combination of SOD and catalase (300 U/ml both; *g* and *h*), the disappearance of  $\alpha$ -catenin and plakoglobin was not inhibited. Treatment of resting EC with a combination of elastase and cathepsin G (1  $\mu$ g/ml both; *i* and *j*) for 5 min at 37°C did not change  $\alpha$ -catenin and plakoglobin localization at EC junctions. Comparable observations were made by staining EC with VE-cadherin and  $\alpha$ -catenin antibodies. (Arrowheads) Adherent PMN. Bar, 10  $\mu$ m.

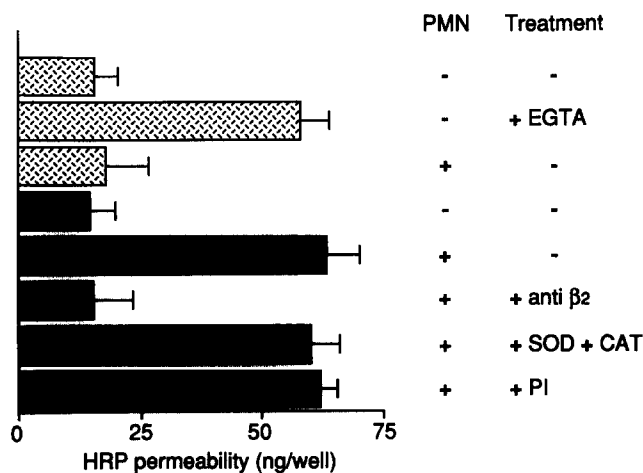
disruption. ICAM-1 clustering triggers tyrosine phosphorylation of cytoskeletal proteins possibly relevant in AJ assembly, such as cortactin and src (Durieu-Trautmann et al., 1994). However, in preliminary experiments when we induced clustering of ICAM-1 by ICAM-1 antibody-coated beads, or by adding ICAM-1 antibodies followed by secondary IgG, we did not observe any change in endothelial AJ organization (not shown). In addition, lymphocyte adhesion to EC, also largely mediated by ICAM-1 (Springer, 1994), did not change AJ. Overall, this data suggest that during PMN adhesion and/or interaction with ICAM-1, some additional mechanisms are necessary for the observed changes in endothelial AJ to take place.

Other authors (Huang et al., 1993; Pfau et al., 1995) have reported that during the adhesion of PMN and natural killer cells, EC cytosolic  $\text{Ca}^{2+}$  level was increased, and addition of intracellular  $\text{Ca}^{2+}$  scavengers blocks PMN transmigration. A possible model is that PMN adherence

could induce a series of EC intracellular responses that cause detachment of catenins from VE-cadherin. The cytoplasmic-free catenins have a short half-life (Kowalczyk et al., 1994), and they are usually destroyed within few minutes. This would be consistent with the parallel disappearance of catenins from both VE-cadherin complex and total cell extracts reported here.

Biologically active intracellular proteins are usually degraded by lysosomes, the ubiquitin-proteasome system, or calpain-dependent proteolysis (Ciechanover, 1994; Saido et al., 1994). A possibility is that the increase in cytosolic  $\text{Ca}^{2+}$  could activate calpain, which in turn could be responsible for VE-cadherin/catenin complex dissociation and catenin lysis. This might be a mechanism similar to that observed by other authors who have reported that the N-cadherin cytoplasmic tail could be partially digested by calpain (Covault et al., 1991).

An alternative explanation for AJ disorganization is

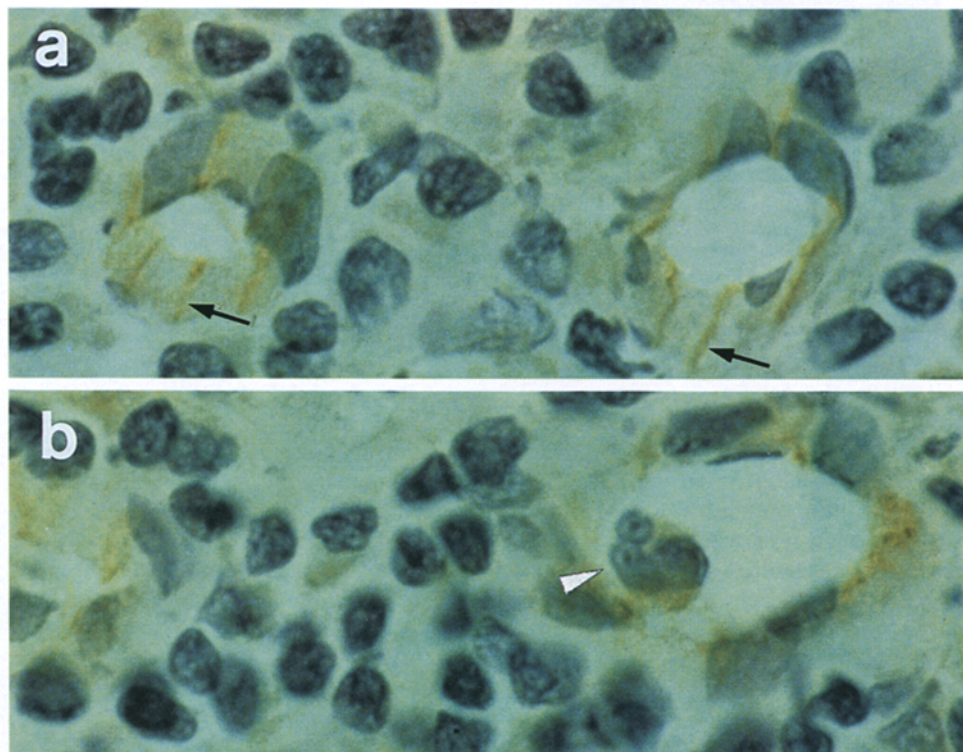


**Figure 11.** PMN adhesion increases EC permeability. EC monolayers were grown to confluence on Transwell inserts, and then exposed for 20 h to 100 U/ml TNF (solid bars) or to control medium (grid bars). Before addition to EC, PMN were treated with TS1/18 mAb (1:100 final dilution, for 20 min: +anti  $\beta_2$ ) or SOD and catalase (300 U/ml both, for 20 min: +SOD+CAT), or with a cocktail of protease inhibitors (100 U/ml aprotinin, 500  $\mu$ M leupeptin, 30  $\mu$ g/ml eglin C, for 30 min: +PI). These agents were kept in the culture medium during EC permeability assay. Permeability of EC monolayers to HRP was tested at 60 min. HRP enzymatic activity was measured as described (Ortiz de Montellano et al., 1988; Navarro et al., 1995). Resting EC, in the presence or absence of resting PMN, presented low permeation to HRP. Addition of EGTA increased permeability by threefold. TNF treatment, per se, did not increase EC permeability, but addition of PMN induced a marked increase in the passage of HRP that was counteracted by the anti- $\beta_2$  mAb but not by SOD in combination with catalase or by protease inhibitors.

that PMN locally release lytic enzymes that can in turn be responsible for VE-cadherin digestion and junction disassembly. This is unlikely, however, since VE-cadherin remains in large part on the cell membrane, and catenin digestion seems to be essentially an intracellular process. In addition, protease inhibitors were ineffective in blocking the action of PMN on AJ and vice versa; addition of elastase and cathepsin G to the culture medium did not modify endothelial junctions. Moreover, the lack of effect of superoxide scavengers strongly suggests that also these metabolites, even if potentially toxic for EC (Sacks et al., 1978; Weiss et al., 1981), do not play a major role in disorganizing endothelial AJ in our experimental conditions.

We cannot exclude that adhesion of PMN could create a microenvironment where active toxic mediators are generated and protected by inhibitors present in the culture medium. It has been shown recently that activated PMN can express a surface-bound elastase and cathepsin G that are particularly resistant to serum inhibitors (Owen et al., 1995). These membrane-bound enzymes or other active components could signal to endothelial cells and induce intracellular modifications leading to AJ disruption. However, for the expression of these membrane-bound enzymes, PMN should be strongly activated (Owen et al., 1995). Here we describe AJ disappearance even with resting PMN, either adherent to TNF-treated EC or after addition of an anti- $\beta_2$ -activating mAb that causes little PMN activation and degranulation (Ortlepp et al., 1995). Furthermore, addition of activated PMN membrane fragments to EC was without effect.

An important question is what is the biological significance of PMN-induced AJ disorganization. We have previously observed that agents that increase endothelial permeability, such as thrombin or EGTA, quickly disrupt



**Figure 12.** PMN adhesion to the vessel wall in vivo changes VE-cadherin distribution at endothelial cell-to-cell contacts. Human tonsil site of chronic recurrent tonsillitis immunostained for VE-cadherin. In high endothelial venules without trafficking leukocytes, VE-cadherin is expressed at the intercellular borders (black arrows). In the area where a PMN is sticking (white arrowhead), the immunostaining of VE-cadherin is reduced, and it is no longer detectable at cell-to-cell contacts. (a and b) Two different areas from the same immunostained typical section. Avidin-biotin-peroxidase complex counterstained with haematoxylin.



endothelial AJ organization (Lampugnani et al., 1992; Rabiet et al., 1996). In addition, truncation of the cytoplasmic tail of VE-cadherin, to prevent its binding to catenins, increases monolayer permeability to high molecular weight molecules (Navarro et al., 1995). It is therefore conceivable that the disruption of VE-cadherin/catenin complex could be associated with an increase in endothelial cell permeability observed in vivo during PMN infiltration in inflamed areas (Wedmore and Williams, 1981; Yi and Ulrich, 1992). We report here, by an in vitro assay, that PMN adhesion to EC increases permeability in a way that is comparable to EGTA. EGTA could be considered a suitable positive control since, in a similar way to PMN adhesion, this agent causes a rapid and complete disappearance of AJ components without changing PECAM-1 distribution or inducing EC retraction (Ayalon et al., 1994; Dejana et al., 1995). Furthermore, as for AJ disruption, proteases and superoxide inhibitors did not change PMN-induced increase in permeability, while the integrin  $\beta_2$ -blocking mAb did so.

Another relevant point is whether, besides permeability, AJ disorganization could facilitate PMN transmigration. Even if we still do not have direct evidence for this argument, in preliminary experiments, we observed that EGTA treatment of EC monolayers increased PMN random passage (Del Maschio, A., A. Zanetti, and E. Dejana, manuscript in preparation). This observation, albeit inconclusive, is suggestive for this possibility.

In conclusion, this work shows that PMN adhesion could trigger VE-cadherin/catenin complex disorganization in EC. This effect seems to be also detectable in vivo. We suggest that AJ disruption could be important in mediating key steps of acute inflammatory reaction, such as vascular oedema and PMN infiltration at sites of microorganism invasion.

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