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VE-cadherin antibody accelerates neutrophil recruitment in vivo

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SUMMARY

Neutrophils enter sites of inflammation by crossing the endothelial lining of the blood vessel wall. VE-cadherin is an endothelial specific, homophilic adhesion molecule located at the lateral cell surface. We have generated a monoclonal antibody against mouse VE-cadherin which inhibits electrical resistance of endothelial cell monolayers in vitro as well as aggregation of VE-cadherin transfected cells. In vivo, this antibody was found to increase vascular permeability and to accelerate the entry of neutrophils into chemically inflamed mouse peritoneum. Thus, VE-cadherin is essential for the integrity of the endothelial barrier in vivo. Our data suggest that opening of VE-cadherin mediated endothelial cell contacts may be a relevant step during neutrophil extravasation.

Key words: Inflammation, Transendothelial migration, Cell adhesion

INTRODUCTION

Exit of neutrophils from the blood stream is initiated by a cascade of cell adhesion molecules and leukocyte activating mediators which control the attachment of neutrophils to the apical surface of endothelial cells (Springer, 1994). Selectins mediate the initial attachment and rolling of leukocytes along the endothelium, while subsequent firm adhesion is based on interactions between activated leukocyte integrins and endothelial adhesion molecules of the Ig-superfamily. Platelet endothelial cell adhesion molecule (PECAM)-1, probably mediates heterotypic adhesive interactions that are crucial for the transendothelial migration of neutrophils, since antibodies against PECAM-1 block neutrophil transmigration after the tight adhesion stage (Muller et al., 1993; Vaporciyan et al., 1993; Bogen et al., 1994).

Transendothelial migration requires mechanisms which open the endothelial cell layer. While such mechanisms have not yet been defined, one possibility is the opening of the endothelial cell junctions. Cadherins are cell adhesion molecules which are involved in the formation and maintenance of junctional cell contacts, as was established for E-cadherin in epithelial cells (Vestweber and Kemler, 1985; Takeichi, 1991; Kemler, 1992). In endothelial cells, several cadherins are expressed, of which only VE-cadherin (cadherin 5) is specific for endothelium while N-cadherin and P-cadherin are also found in other cell types (Suzuki et al., 1991; Liaw et al., 1990; Lampugnani et al., 1992; Breier et al., 1996). VE-cadherin is concentrated at interendothelial cell contacts and is involved in the maintenance of cell layer integrity of human endothelial cell cultures (Lampugnani et al., 1992; Navarro et al., 1995). The function of VE-cadherin in an intact animal model has not yet been determined.

We have examined whether VE-cadherin would be necessary for the maintenance of cell contacts between endothelial cells in vivo. Assuming that neutrophils would be able to exit from blood vessels through the paracellular route between endothelial cells, we tested whether antibodies against VE-cadherin would influence neutrophil extravasation.

MATERIALS AND METHODS

VE-cadherin-Ig fusion protein

A VE-cadherin-IgG fusion protein was generated by placing a cDNA fragment coding for the complete extracellular part of mouse VE-cadherin including the membrane proximal glutamine in front of a cDNA fragment coding for the Fc part of human IgG1, including the hinge region and immunoglobulin domains C2 and C3. Construction of the expression plasmid, cell transfection and production of the fusion protein was done essentially as described (Hahne et al., 1993a; Vestweber, 1996).

Antibodies

For generation of monoclonal antibodies against mouse VE-cadherin, Lewis rats were immunized with the mouse VE-cadherin-Ig fusion protein. Immunizations and mAb screening and production was performed as described for the anti-P-selectin antibody (Bosse and Vestweber, 1994). Five monoclonal antibodies were found which recognized VE-cadherin-IgG but not the control proteins E-selectin-IgG or P-selectin-IgG (Hahne et al., 1993a) in ELISA assays. One of these antibodies was the rat IgG_{2a} 11D4.1. The non-blocking monoclonal antibody 9DB3 against mouse VCAM-1 has been described (Hahne et al., 1993b). Rabbit antisera were raised against the peptide CSD-

PQEELII covering the C terminus of mouse VE-cadherin or the peptide CKKHVNPVQALSEFK covering the C terminus of mouse α-catenin. Peptides were conjugated to a carrier protein and used for immunization as described (Weller et al., 1992). The rabbit antiserum against mouse N-cadherin against an N-cadherin containing bacterial fusion protein has been described (Herrenknecht et al., 1991). Immunoprecipitations were done as described (Levinovitz et al., 1993; Lenter and Vestweber, 1994).

Cells

Mouse bEnd.3 endothelioma cells had been established from mouse brain capillaries and were kindly provided by Dr Werner Risau (Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim, Germany). These cells as well as mouse endothelioma cells eEnd.2 (Williams et al., 1988) were cultured in DMEM with 10% fetal calf serum (FCS). CHO cells DUKX B1 were obtained from the American Type Culture Collection and cultured in αMEM medium supplemented with 10% FCS. Medium for transfected CHO cells was supplemented with 800 μg/ml G418.

Transfection and stable expression of VE-cadherin

CHO DUKX B1 cells were electroporated with pcDNA3 containing either no insert or the cDNA for mouse VE-cadherin (Breier et al., 1996). In brief, 1×10^7 CHO cells resuspended in 600 μl PBS containing 5 μg of plasmid DNA in a 0.4 cm cuvette were electroporated at 950 μF and 0.25 kV. Transfected cells were selected in the presence of 800 μg/ml of G418 (Sigma) and individual cell clones of each transfectant were analysed for VE-cadherin expression by flow cytometry as described (Levinovitz et al., 1993). First antibodies were detected with FITC labeled second stage antibodies from Dianova (Hamburg, Germany).

Transendothelial electrical resistance assay

Mouse bEnd.3 endothelioma cells were seeded at a density of 4×10^4 cells onto laminin (Sigma, 50 μg/ml) coated cell culture inserts (Falcon 3095; 0.45 μm pore size) and cultured in DMEM containing 10% FCS. The medium was renewed every other day and electrical resistance was monitored daily at 22°C with a Ag/AgCl-electrode and compared to a control filter without cells. In order to examine cell density and even distribution of cells on the filters endothelial cell monolayers were stained with Dade-Diff-Quik (Baxter, Düdingen, Schweiz) according to the manufacturer's instructions.

Cell aggregation assay

Mouse VE-cadherin transfected CHO cells or mock-transfected CHO cells were harvested with trypsin in the presence of Ca^{2+}, washed and allowed to aggregate under rotation (80 rpm) for 90 minutes at 37°C at a density of 6×10^5 cells/ml in Hanks' buffered salt solution (containing 25 mM Hepes and 5 mM $CaCl^{2+}$). Cells were pre-incubated for 30 minutes on ice prior to the assay either with 200 μg/ml control antibody or 200 μg/ml mAb 11D4.1 (11D4). The assay was evaluated by counting the numbers of particles using a Coulter Epics XL FACS-sorter.

In vivo peritonitis model

Experiments were essentially performed as described (Bosse and Vestweber, 1994). Briefly, female 8- to 10-week-old Balb/c mice (Charles River Wiga, Sulzfeld, Germany) were injected intravenously with 200 μl PBS, either containing no antibody or 20 μg of antibody 11D4.1 against mouse VE-cadherin or 20 μg of the non-blocking control antibody 9DB3 against mouse VCAM-1. Antibody injection was immediately followed by intraperitoneal administration of 1 ml thioglycollate-boullion (Merck, Darmstadt, Germany). At different time points after stimulation, cells were harvested from peritoneum and the total number of emigrated neutrophils was determined in the following way. Mice were killed at indicated time points and peritoneal lavage was performed by injecting 10 ml PBS, massaging the peritoneal wall and removing the fluid. Total cell numbers were determined in a hemocytometer. Cytospin preparations were made, fixed in methanol and stained with Dade-Diff-Quik (Baxter, Düdingen, Schweiz) according to the manufacturer's instructions. Cells were differentially counted by microscopy, evaluating 300 cells per slide. From the total cell count in the peritoneal lavage and the percentage of neutrophils determined from cytospin preparations the absolute number of neutrophils in the peritoneal lavage was calculated.

RESULTS

In order to analyse a possible function of VE-cadherin as a barrier for leukocyte extravasation, we raised a cell adhesion blocking monoclonal antibody (mAb) against mouse VE-cadherin. We immunized rats with a VE-cadherin-Ig fusion protein containing the complete extracellular part of mouse VE-cadherin, placed in front of the hinge region of human IgG_1. Five monoclonal antibodies were found which recognized VE-cadherin-Ig in ELISA assays, but not various other Ig-chimeras. One of them, the mAb 11D4.1 (rat IgG_{2a}) immunoprecipitated proteins of 125, 102 and 88 kDa from metabolically labeled mouse endothelioma cells (Fig. 1, lane 2). These proteins had identical electrophoretic mobilities to those of VE-cadherin and the associated cytoplasmic proteins α- and β-catenin, as was confirmed in immunoprecipitations with affinity purified polyclonal rabbit antibodies against peptides of mouse VE-cadherin and α-catenin, respectively (Fig. 1, lanes 3 and 6). The antibody 11D4.1 did not cross-react with N-cadherin which was also present in these cells, as detected with polyclonal anti-N-cadherin antibodies (Fig. 1, lane 4).

In order to examine a possible adhesion blocking activity of mAb 11D4.1 we decided to perform cell aggregation assays with VE-cadherin transfected CHO cells. To this end, CHO cell clones were generated which were permanently transfected with mouse VE-cadherin. As shown in Fig. 2 surface expression of VE-cadherin on one of these transfected CHO clones was analysed with mAb 11D4.1 by flow cytometry.

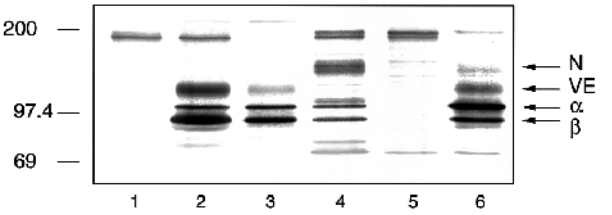

Fig. 1. MAb 11D4.1 immunoprecipitates VE-cadherin. Mouse endothelioma cell line eEnd.2 was metabolically labeled with [^{35}S]methionine and detergent extracts were immunoprecipitated with rat IgG as a control (lane 1) rat mAb 11D4.1 (lane 2), affinity purified polyclonal rabbit IgG against a peptide of mouse VE-cadherin (lane 3), polyclonal rabbit IgG against mouse N-cadherin (lane 4), rabbit IgG of a non-immune serum (lane 5) or affinity purified polyclonal rabbit IgG against a peptide of mouse α-catenin (lane 6). Arrows on the right denote the positions of N-cadherin (N), VE-cadherin (VE), α-catenin (α) and β-catenin (β). Molecular mass markers (in kDa) are indicated on the left. Note that mAb 11D4.1 did not crossreact with N-cadherin and that both cadherins were found to be associated with α- and β-catenin.

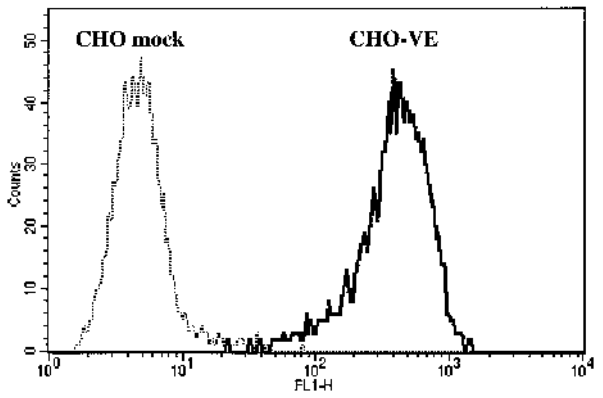

Fig. 2. Cell surface expression of VE-cadherin in CHO transfectants. Flow cytometric analysis of mock transfected CHO cells (CHO mock) and of a VE-cadherin-transfected CHO cell clone (CHO-VE) with monoclonal antibody 11D4.1. First antibody was detected with a FITC labeled second stage antibody.

In cell aggregation assays with the VE-cadherin transfected clone and with mock transfected CHO cells single cell suspensions were incubated for 90 minutes at 37°C rotating at 80 rpm. Mock tansfected cells did not aggregate, while VE-cadherin transfected cells aggregated in large clumps (Fig. 3). This aggregation was largely inhibited in the presence of mAb 11D4.1, but not in the presence of an isotype matched control antibody (Fig. 3).

The inhibitory effect of mAb 11D4.1 on cell contacts between endothelial cells was analysed by testing the effect of the antibody on the electrical resistance across a monolayer of mouse endothelioma bEnd.3 cells grown on laminin coated transwell filters. Cells were grown in the presence of 20 μg/ml 11D4.1 or, as controls, the same amount of the non-blocking antibody 9DB3 (Hahne et al., 1993b) against mouse VCAM-1 or the mAb EA-3 against mouse PECAM-1 (Piali et al., 1993). In all cases the endothelial cell monolayers reached confluency. Cell density and an even distribution of the cells on the filters was analysed by staining the cells with Dade-Diff-Quik and examining them microscopically (data not shown). Although cell density of the cells was not affected the electrical resistance of cell monolayers grown in the presence of 20 μg/ml 11D4.1 was reduced for more than 50% compared to monolayers cultured without antibodies or in the presence of the control antibodies (Fig. 4). Thus, mAb 11D4.1 inhibits the junctional integrity of mouse endothelial cells in vitro.

We next examined, whether intravenous injection of mAb 11D4.1 into mice would alter neutrophil accumulation induced by application of thioglycollate into the peritoneal cavity. Antibodies (20 μg) were intravenously injected at the time of intraperitoneal injection of thioglycollate. As shown in Fig. 5A, maximal levels of infiltrated neutrophils in the peritoneum were observed 8 hours after thioglycollate-stimulation in control animals which had been treated with the non-blocking, isotype matched control antibody 9DB3 against mouse VCAM-1. This antibody binds to activated endothelium (Hahne et al., 1993b) and has no effect on the entry of neutrophils into inflamed peritoneum (Bosse and Vestweber, 1994; and data not shown). Cell numbers decreased again over a period of the next 16 hours. In contrast, neutrophils in 11D4.1 treated animals accumulated much faster in the peritoneum, reaching maximal levels already as early as 4 hours after stimulation with thioglycollate (Fig. 5A). The maximal numbers of infiltrated neutrophils at 4 hours was more than three times higher than in mice treated with control antibodies (Fig. 5A). This is also illustrated by the stained cytospin preparations of equal aliquots of the peritoneal PBS lavage from mice stimulated for four hours with thioglycollate and intravenously injected either with control antibody 9DB3 or the anti-VE-cadherin

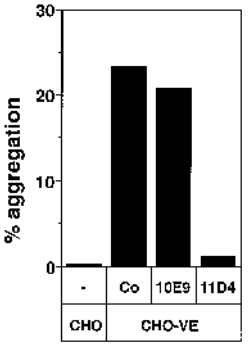

Fig. 3. Aggregation of VE-cadherin-transfected CHO cells is blocked by anti-VE-cadherin antibody. Mock-transfected CHO cells (CHO) or mouse VE-cadherin transfected CHO cells (CHO-VE) were allowed to aggregate after pre-incubation either without antibody (Co), with control antibody 10E9 against mouse E-selectin (10E9) or with mAb 11D4.1 (11D4). Specific aggregation was calculated by dividing the difference between the particle number before and after the assay by the number of cells with which the assay was started.

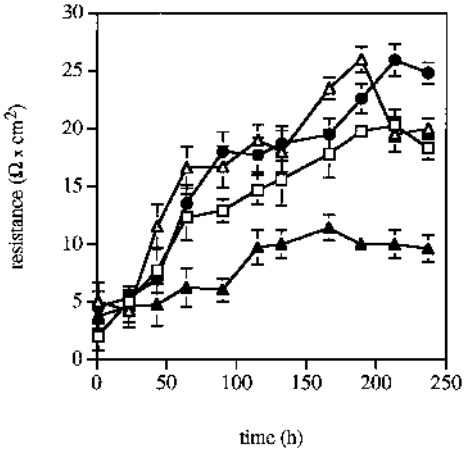

Fig. 4. VE-cadherin antibody reduces electrical resistance of mouse endothelioma cell monolayers on transwell filters. Mouse brain derived endothelioma cells bEnd.3 were cultured for 10 days on transwell filters in the absence of antibodies (△), or in the presence of mAb 9DB3 (Hahne et al., 1993b) against mouse VCAM-1 (□), mAb EA-3 (Piali et al., 1993) against mouse PECAM-1 (●), or mAb 11D4.1 against mouse VE-cadherin (▲). All antibodies were present at a concentration of 20 μg/ml and were renewed every other day. All antibody preparations were essentially free of endotoxin contamination. Data represent the average electrical resistance of monolayers ($\Omega \times cm^2$) ± s.e.m. for triplicates. Results shown are representative for three separate experiments.

antibody (Fig. 5B). As in control animals, the accumulation of neutrophils was transient. The generation of Fab fragments of 11D4.1 proved technically problematic. However, the failure of the anti-VCAM-1 control antibody to influence emigration rules against non-specific or Fc-mediated effects of bound mAb being responsible for the enhanced effect seen with the anti-VE-cadherin mAb. Circulating white blood cell counts were determined for each time point and showed no significant changes from baseline counts.

In order to test whether 11D4.1 can increase vascular permeability 50 μg of the antibody was injected intravenously followed 1 hour later by intravenous injection of the dye Evan's Blue (100 μl, 0.1% solution). Thirty minutes after injection of the dye the illiosacral lymph nodes were the first sites which were stained blue in the 11D4.1 treated animals, while no effect was seen in PBS-treated, Evans Blue injected control mice. Examination of the histology of these lymph nodes did not allow us to detect any sign of general disruption of the endothelium (data not shown). Thus, the anti-VE-cadherin antibody can increase the permeability of blood vessels. These data suggest that the accelerating effect of the anti-VE-cadherin antibody on neutrophil extravasation is probably due to interference with the integrity of endothelial cell contacts and not due to an indirect effect, stimulating the capability of endothelial cells to support transmigration of leukocytes.

DISCUSSION

In this study, we have analysed the effect of a monoclonal antibody against mouse VE-cadherin on the integrity of endothelial cell contacts in vivo. We have generated a monoclonal antibody which inhibits aggregation of VE-cadherin transfectants and blocks the establishment of electrical resistance across an endothelial cell monolayer in vitro. When injected intravenously, this antibody accelerated neutrophil migration into inflamed peritoneum. In addition, the antibody increased the permeability of blood vessels for the dye Evans Blue. Our data suggest that VE-cadherin mediated endothelial cell contacts represent a barrier for extravasating neutrophils. Opening this barrier allows neutrophils to exit from blood vessels more rapidly.

How the antibody interferes with the integrity of the endothelial cell layer in vivo, could be explained in two ways. First, the antibody could directly compete with the binding between VE-cadherin molecules on adjacent cells. Similar effects have been observed for the effect of anti-E-cadherin antibodies on the integrity of epithelial cell layers in vitro (Vestweber and Kemler, 1985). While in the case of polarized epithelium, intact tight junctions prevent the accessibility of E-cadherin for reagents added from the apical side of the monolayer, the situation in postcapillary venules is different. The endothelium of these blood vessels, which represents the major sites for

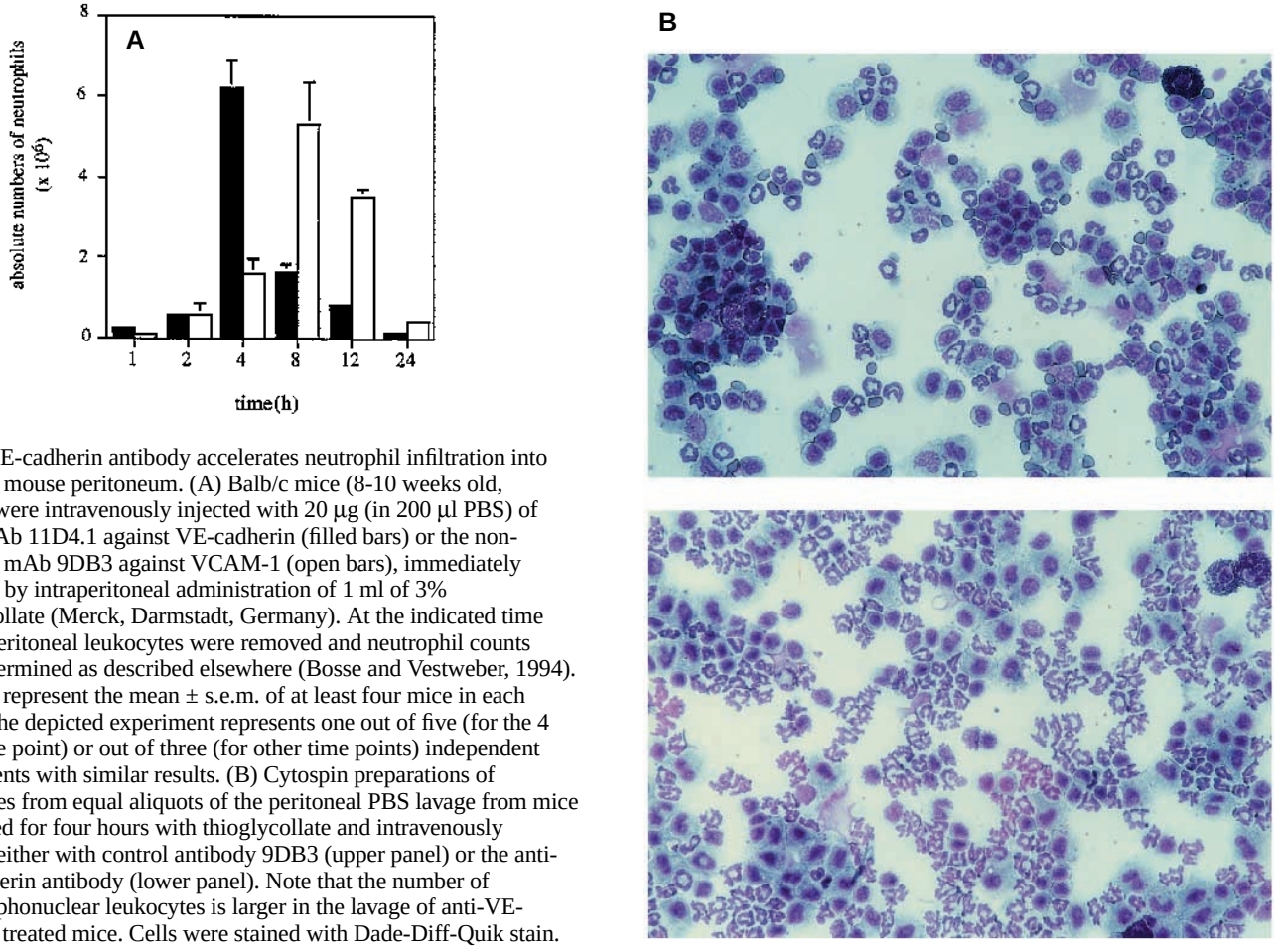

Fig. 5. VE-cadherin antibody accelerates neutrophil infiltration into inflamed mouse peritoneum. (A) Balb/c mice (8-10 weeks old, female) were intravenously injected with 20 μg (in 200 μl PBS) of either mAb 11D4.1 against VE-cadherin (filled bars) or the non-blocking mAb 9DB3 against VCAM-1 (open bars), immediately followed by intraperitoneal administration of 1 ml of 3% thioglycollate (Merck, Darmstadt, Germany). At the indicated time points, peritoneal leukocytes were removed and neutrophil counts were determined as described elsewhere (Bosse and Vestweber, 1994). The data represent the mean ± s.e.m. of at least four mice in each group. The depicted experiment represents one out of five (for the 4 hour time point) or out of three (for other time points) independent experiments with similar results. (B) Cytospin preparations of leukocytes from equal aliquots of the peritoneal PBS lavage from mice stimulated for four hours with thioglycollate and intravenously injected either with control antibody 9DB3 (upper panel) or the anti-VE-cadherin antibody (lower panel). Note that the number of polymorphonuclear leukocytes is larger in the lavage of anti-VE-cadherin treated mice. Cells were stained with Dade-Diff-Quik stain.

leukocyte extravasation, is basically devoid of tight junctions (Simionescu and Simionescu, 1991; Dejana et al., 1995), which would allow accessibilty of VE-cadherin from the luminal side.

A second, alternative, explanation for the mode of action by which the anti-VE-cadherin antibody interferes with cell contacts could be that it blocks the closing of endothelial cell contacts after transmigration of the first neutrophils, rather than directly opening VE-cadherin mediated contacts. The fact, that we were unable to reduce the electrical resistance across cultured endothelial cell monolayers by adding the antibody days after an intact monolayer had been formed argues for this second explanation. Although, the inability to reduce electrical resistance under these conditions could also be explained by the formation of tight junctions between the bEnd cells which prevent accessibility of VE-cadherin from the apical side. In this context it is noteworthy that lymph node tissue was the first where leakiness for the dye Evans Blue was observed after intravenous application of the anti-VE-cadherin antibody. In lymph nodes, which are more active in leukocyte traffic than other organs, the mAb against VE-cadherin may have a better chance to interfere with the closing of endothelial cell contacts after leukocyte extravasation.

It is important to note that the effect of the mAb against VE-cadherin on the integrity of the endothelial barrier in vivo was subtle and did not accelerate extravasation of just any type of leukocytes. Usually mononuclear cells enter inflamed peritoneum 24 to 48 hours after neutrophils have massively accumulated. The fact that the antibody did not induce massive accumulation of mononuclear cells at 4 hours after thioglycollate stimulation (not shown) indicates, that leukocyte extravasation was still dependent on specific mechanisms, most likely chemotactic stimuli which can distinguish between neutrophils and mononuclear cells.

Despite the simple composition of the interendothelial junctional complex in postcapillary venules, the in vivo effect of our anti-VE-cadherin mAb suggests that these junctions represent a barrier for extravasating neutrophils. VE-cadherin seems to mediate an essential adhesion mechanism which stabilizes this barrier, since blocking its function is sufficient to induce vascular permeability and to enhance neutrophil extravasation. Our data do not exclude the possibility that extravasating leukocytes leave blood vessels via the transcellular route by directly penetrating through single endothelial cells (Hammersen and Hammersen, 1987; Neal and Michel, 1992; Farr and DeBruyn, 1975; Faustmann and Dermietzel, 1985; Cho and DeBruyn, 1981; Lossinsky et al., 1991). However, our results support the concept that neutrophils extravasate through the paracellular route.

It will be important to elucidate possible mechanisms which may stimulate the opening of VE-cadherin mediated cell contacts following leukocyte attachment to endothelium. Indeed, it has been shown recently (Del Maschio et al., 1996), that docking of neutrophils to the apical surface of cultured human endothelial cells causes degradation of VE-cadherin associated β-catenin and leads to increased permeability of the endothelial cell monolayer for macromolecules. Regulation of endothelial cell contacts may well be an additional regulatory step in the already known cascade of steps which control leukocyte extravasation.

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