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Locking endothelial junctions blocks leukocyte extravasation, but not in all tissues

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The passage of leukocytes across the blood vessel wall is a fundamental event in the inflammatory response. During the last decades, there has been significant progress in understanding the molecular mechanisms involved in leukocyte transmigration. However, it is still a matter of debate whether leukocytes migrate paracellularly or transcellularly through an endothelial cell layer. We could recently show that a VE-cadherin-α-catenin fusion protein locks endothelial junctions in the skin and strongly reduces leukocyte diapedesis in lung, skin and cremaster, establishing the paracellular route as the major transmigration pathway in these tissues. However, the homing of naïve lymphocytes into lymph nodes and extravasation of neutrophils in the inflamed peritoneum were not affected by VE-cadherin-α-catenin. This unexpected heterogeneity of the diapedesis process in different tissues as well as the complexity and dynamics of the cadherin-catenin complex in regulating endothelial junctions will be discussed.

Keywords: leukocyte trafficking, diapedesis, endothelial junctions, VE-cadherin, cell adhesion

Abbreviations: For full list of abbreviations, see page 5

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Two Routes of Leukocyte Transendothelial Migration

The migration of leukocytes through a barrier of endothelial cells, called diapedesis, is a key process of the immune response. It has been studied for decades whether leukocytes migrate transcellularly through the body of endothelial cells or paracellularly through the junctions between adjacent endothelial cells. Based on electron microscopy numerous reports have presented evidence for transcellular migration,[1-4] whereas many others clearly demonstrated paracellular migration events.[5-7] Most of these studies used serial sectioning followed by electron microscopy. Due to this tedious and laborious procedure only very few leukocytes could be analyzed in each study, leaving the question of the quantitative relevance of each of the two pathways unanswered. More recently, 3D live imaging by confocal microscopy of neutrophils extravasating in the inflamed cremaster muscle revealed that about 90% of all neutrophils used the paracellular pathway.[8] However, even with this outstanding technology it is difficult to distinguish in each case whether a given leukocyte is indeed migrating through a junction or simply very close to a junction, but actually starting the process by a transcellular mechanism.[9] Furthermore, this elaborated high resolution 3D imaging still needs to be developed for other organs to be more generally applicable for a large variety of tissues.

In vitro transmigration assays allow a much easier distinction of the para- and trans-cellular diapedesis events. Interestingly these studies suggest that there is variation depending on the type of leukocytes and endothelial cells studied.[10-13] For peripheral blood mononuclear cells it was reported that they mainly use the transcellular migration route through a HUVEC monolayer under flow conditions, whereas neutrophils were found to use only the junctional pathway.[12,13] In the absence of flow, 5–11% of leukocytes crossed the HUVEC monolayer transcellularly,[11] while employing microvascular

endothelial cells increased the fraction of these cells to about 30%.[10]

In order to study the diapedesis process in vivo in a variety of tissues independently of technical limitations of microscopic procedures we decided to use a genetic approach. We generated mice with modified endothelial junctions that should resist opening and asked whether this would affect the leukocyte extravasation process. The advantage of this approach was that we could analyze the leukocyte extravasation process in a variety of tissues and organs.[14]

Stabilization of the VE-Cadherin-Catenin Complex Can Lock Endothelial Junctions and Inhibit Transendothelial Migration

VE-cadherin mediated adhesion has been shown to be a key factor in regulating endothelial barrier function. It was reported that adhesion-blocking antibodies against VE-cadherin can destabilize endothelial junctions in vivo,[15,16] suggesting that the adhesive function of VE-cadherin is essential for the stability of endothelial cell contacts. Cadherin adhesive strength in the endothelium is dependent on the association with the catenins. The VE-cadherin cytoplasmic domain directly binds to β-catenin, which in turn associates with α-catenin. α-catenin links the whole complex, probably indirectly, to the actin cytoskeleton and is indispensable for cadherin-mediated adhesion.

In vitro studies have revealed that an E-cadherin-α-catenin fusion protein transfected into fibroblasts or erythroleukemia cells mediates much more stable cell adhesion than E-cadherin alone.[17,18] This stronger adhesion could even resist a treatment with peroxyvanadate,[18] a general phosphatase inhibitor known to disrupt cell adhesion due to enhancing tyrosine phosphorylation of junctional proteins. Based on these results, we tried to stabilize the adhesive function of VE-cadherin by constructing an analogous molecule, fusing VE-cadherin lacking its β-catenin binding site to a truncated form of α-catenin comprising the C-terminal two-thirds of the protein. To test the effect of this protein on endothelial junctions in vivo, we generated transgenic mice replacing wild type VE-cadherin with the VE-cadherin-α-catenin (VEC-α-C) fusion molecule by specifically targeting the endogenous VE-cadherin (*Cdh5*) gene locus.[14] About half of the homozygous mice on a mixed C57/BL6 / SV129 genetic background were viable and fertile and exhibited no obvious defects. The phenotype of those embryos that die during development is presently under investigation. Western blots of lung homogenates of homozygous adult animals revealed essentially similar levels of VEC-α-C expression compared with VE-cadherin levels in wild type mice (VEC-WT). We could show that the VEC-α-C fusion protein was located at cell-cell contacts and that the amount and distribution of other molecules important for endothelial cell-cell contacts was unchanged. Different inflammation models were employed to examine the effects of the VEC-α-C fusion molecule on endothelial cell-cell contacts in vivo. Using the Miles vascular permeability assay, we could show that VEC-α-C caused a complete block of VEGF- and histamine-induced vascular permeability in the skin. Thus, we can conclude that VEC-α-C is indeed able to strongly stabilize endothelial junctions and that weakening of adherens junctions is an essential step during induction of vascular permeability.

We then examined the extravasation of leukocytes, and found that neutrophil diapedesis was reduced by up to 74% in the cremaster of VEC-α-C mice. Investigating other tissues, we found that LPS-induced recruitment of neutrophils into the lung was reduced by 63% in VEC-α-C mice compared with control mice, and the emigration of antigen-challenged T cells into the dinitrofluorbenzene (DNFB)-challenged skin was reduced by 57% in a delayed type hypersensitivity (DTH) reaction. Whether residual leukocyte extravasation was due to transcellular diapedesis or whether VEC-α-C mediated stabilization of endothelial junctions could only partially block the opening of junctions by leukocytes can presently not be answered. Independent of this issue we can conclude, that our results provide a minimum estimate for the relevance of the paracellular transmigration route in different tissues in vivo, clearly demonstrating that the paracellular pathway is the major route in these tissues. Importantly, we could verify in vitro that the VEC-α-C fusion protein indeed blocked exclusively the paracellular transmigration route in transmigration assays with cultured endothelial cells, whereas the transcellular pathway was not affected.[14]

VE-cadherin-α-catenin Does Not Inhibit Lymphocyte Homing to Lymph Nodes and Neutrophil Extravasation in the Inflamed Peritoneum

In light of the clear inhibition of leukocyte extravasation in various inflamed tissues, we were surprised to find that homing of naïve lymphocytes into peripheral and mesenteric lymph nodes was not significantly reduced in VEC-α-C mice in comparison to control mice.[14] Furthermore, we found that even neutrophil extravasation in the IL-1β inflamed peritoneum (10 ng, 2 h) was not reduced in VEC-α-C mice (**Fig. 1A**). This unexpected result reveals that leukocyte diapedesis seems to be very differently regulated in different tissues. To exclude that VEC-α-C is not or to a lesser extend expressed in the vessels of the peritoneal cavity, we analyzed whole mount stainings of peritoneal postcapillary venules. We analyzed vessels of the omentum since this has been suggested to be a major site of leukocyte extravasation into the peritoneal cavity.[19,20] Expression of VEC-α-C was indistinguishable from the expression level of VEC-WT and also the tight junction marker ESAM was expressed similarly in both VEC-α-C and VEC-WT mice (**Fig. 1B**). The surprising lack of any inhibitory effect of VEC-α-C in the peritonitis model prompted us to analyze further whether VEC-α-C is generally able to lock endothelial junctions in peritoneal venules in vivo. We performed a peritoneal permeability assay for which we intravenously injected Evan's blue and stimulated intraperitoneally with IL-1β. One hour later we determined the amount of Evan's Blue that had leaked out into the peritoneal cavity. We found that basal vascular permeability in VEC-α-C mice was decreased compared with VEC-WT mice suggesting that VEC-α-C is indeed able to stabilize junctions in this tissue

(**Fig. 1C**). However, the IL-1β induced increase in permeability was not reduced in VEC-α-C mice compared with control mice (**Fig. 1C**). Thus, VEC-α-C is not able to prevent induced opening of endothelial junctions in peritoneal venules. Extrapolated to our leukocyte diapedesis results, this suggests that VEC-α-C may not be able to prevent leukocyte-induced opening of endothelial junctions in peritoneal venules, in contrast to the other tissues we analyzed. Alternatively, we cannot rule out that peritoneal venules may favor extravasation via the transcellular route.

Tissue Specific Mechanisms for Leukocyte Transmigration or Induction of Permeability

The molecular mechanisms of paracellular and transcellular transmigration are not yet understood in detail, particularly regarding the variations in different tissues. The entry of naïve lymphocytes into lymph nodes for example occurs in specialized vessels, the high endothelial venules (HEVs). These vessels are known to provide additional adhesion molecules, carrying the addressins MAdCAM-1 or PNAd, which facilitate extravasation.[21] In addition, the morphology of the endothelial cells in these vessels with their high cuboidal shape is very different from most other endothelial cells in the body. It is possible that endothelial cells of lymph node HEVs provide other molecular mechanisms including additional adhesion or signaling molecules that are able to regulate efficient diapedesis by other means. This might occur either by affecting VE-cadherin in different ways than in other vessels or by circumventing the opening of VE-cadherin supported junctions. For HEVs it has been shown that lymphocytes exit as a row of cells that migrate one after the other through the same opening in an endothelial junction.[5] One lymphocyte that succeeds in opening an endothelial junction despite of VEC-α-C expression could thereby facilitate the exit of the following lymphocytes. Similar mechanisms might be at work in some of the peritoneal blood vessels. The omentum, the largest peritoneal fold, represents a major site of leukocyte entry into the peritoneal cavity and within this tissue the so called milky spots are believed to be the main extravasation sites for leukocytes in the omentum.[20,22] Milky spots are morphologically and functionally distinct structures that possess a specialized blood vasculature and are sites of B- and T-cell and macrophage accumulation. Interestingly, vessels in the center of the milky spots are HEV like vessels that carry PNAd and MAdCAM-1.[23,24] It is tempting to speculate that extravasation through omental milky spots could be

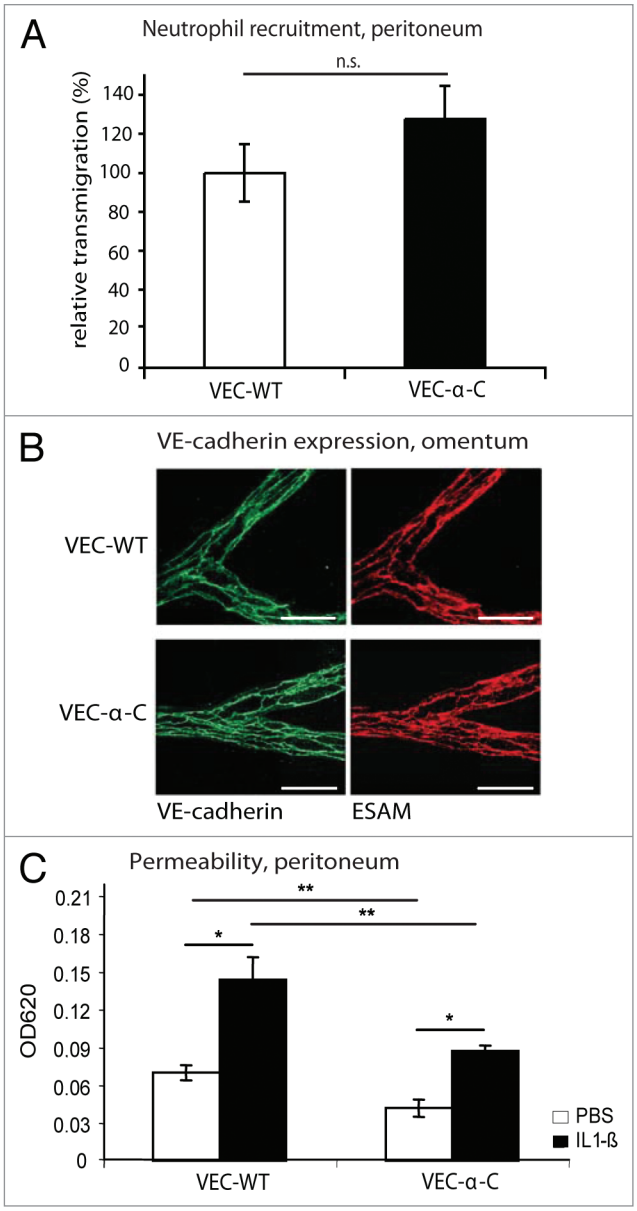

Figure 1. VE-cadherin-α-catenin does neither inhibit IL-1β induced extravasation of neutrophils nor the stimulation of vascular permeability in the peritoneum. (**A**) Peritoneal neutrophil influx 2 h after intraperitoneal injection of PBS for controls (open bars) or 10 ng IL-1β (black bars) in VEC-WT or VEC-α-C mice. At least four mice were analyzed per group. The number of neutrophils emigrated in VEC-WT mice was set to 100%. One representative out of three independent experiments is shown. (**B**) Whole mount stainings of vessels in the omentum of VEC-WT and VEC-α-C mice showing similar expression of VEC-WT and VEC-α-C in cell contacts. Likewise, staining of cell contacts for ESAM was indistinguishable. Projections of z-stacks taken on an LSM Zeiss 510 Meta, bar = 20 μm. (**C**) Vascular leakage of dye into the peritoneal cavity of VEC-WT and VEC-α-C mice 1 h after intravenous injection of Evan's blue and subsequent intraperitoneal injection of PBS or 10 ng IL-1β. Four to six mice were analyzed per group. One representative out of two independent experiments is shown.

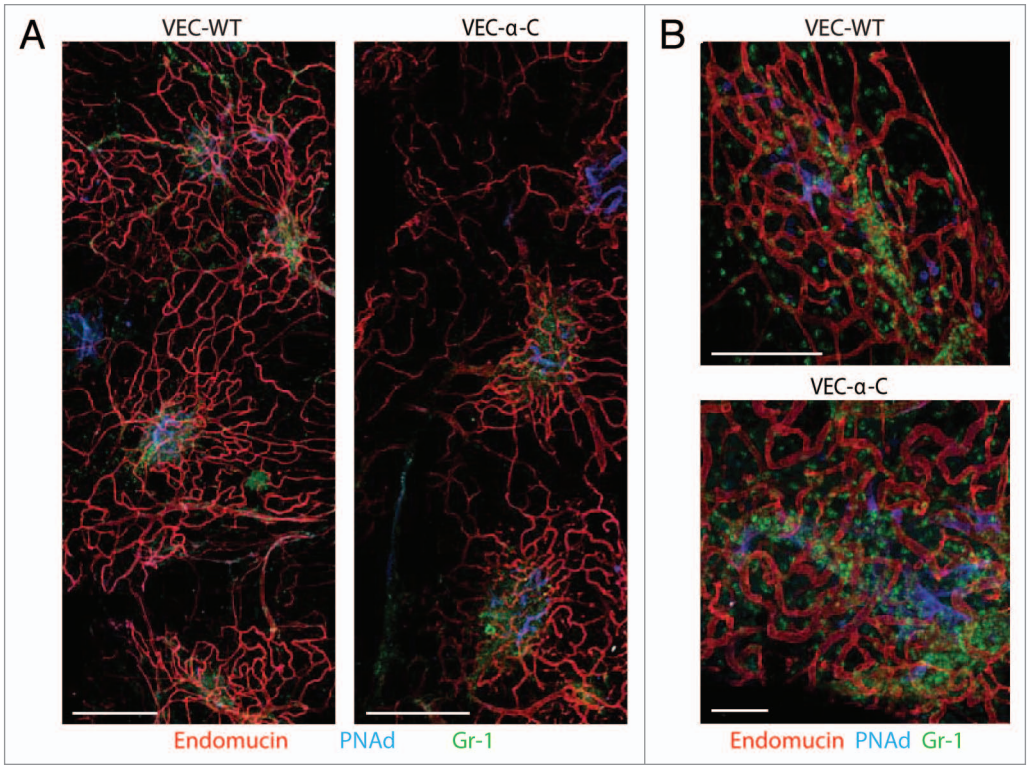

Figure 2. Massive extravasation of neutrophils in the area of milky spots of the IL-1β-inflamed omentum in VEC-WT and VE-cadherin-α-catenin mice. Whole mount stainings of vessels of the omentum of VEC-WT and VEC-α-C mice 1 h after intraperitoneal injection of 10 ng IL-1β. Venous endomucin staining (red), staining of HEV like vessels in milky spots by PNAd (blue) and labeling of extravasated Gr-1 positive neutrophils (green) was comparable in VEC-WT and VEC-α-C mice. Projections of z-stacks taken on an LSM Zeiss 780. Bars = 200 μm (**A**) and 50 μm (**B**).

to some extent similar to the entry of leukocytes into lymph nodes. Indeed, we found massive extravasation of neutrophils in milky spots of the omentum 1 h after intraperitoneal stimulation with IL-1β (**Fig. 2**). This accumulation of extravasated neutrophils was similar in the omentum of VEC-α-C mice and VEC-WT mice (**Fig. 2**). However, while a dominant role of the HEVs in these tissue fragments in leukocyte extravasation is likely, we do not know to which extent other vessels contribute to the recruitment of neutrophils. Thus, it is presently still open to what extent special features of HEVs in milky spots are responsible for the unhampered neutrophil recruitment into the inflamed peritoneum of VEC-α-C mice.

It is remarkable that also other steps of the extravasation process differ between the peritoneum and other tissues. For example, it was found that blocking of ICAM-2 with antibodies significantly suppressed IL-1β induced neutrophil transmigration in PECAM-1 KO animals in the peritonitis model, but not in the cremaster muscle.[25] The magnitude of the effects of different signaling pathways that induce leukocyte integrin activation also seems to vary. Whereas blocking of GPCR signaling with pertussis toxin strongly reduces neutrophil extravasation in a peritonitis model, the same treatment has no effect on the extravasation of neutrophils in the TNFα stimulated cremaster.[26] LSP-1, an intracellular Ca^{2+} and F-actin binding protein in leukocytes and endothelial cells, was found to be relevant in endothelial cells for neutrophil extravasation in the cremaster, but not in peritoneal vessels.[27] This illustrates important variations in the extravasation process in different tissues.

Finally, we also need to consider that another reason why the VEC-α-C fusion protein does not inhibit the diapedesis process in lymph nodes and the peritoneum might be that leukocytes indeed use the transcellular pathway in these tissues. One way to further clarify this would be to use high resolution live 3D imaging on omentum and mesenteries, as it has recently been successfully used for the cremaster.[8]

Molecular Basis for Stabilizing Endothelial Junctions by Fusing VE-cadherin to α-catenin

It is an interesting question why the direct and covalent linkage of VE-cadherin with α-catenin stabilizes endothelial junctions. In our study, we could not detect any indirect effects of the VEC-α-C fusion protein as none of the analyzed endothelial signaling pathways were altered in VEC-α-C mice.[14] In particular, no differences were detected in VEGFR2-signaling and also the cytoplasmic pool of β-catenin was unaffected and Wnt-induced translocation of β-catenin into the nucleus was normal. Furthermore, VEC-α-C association with the phosphatase VE-PTP and with p120-catenin was similar to VEC-WT and also endocytosis of VEC-α-C did not differ from VEC-WT. Notably, only paracellular, but not transcellular transmigration

of leukocytes was inhibited by the fusion of VE-cadherin to α-catenin in vitro. Moreover, expression levels of other cell contact molecules as well as adhesion molecules necessary for leukocyte binding were comparable in VEC-α-C and VEC-WT mice. The only difference that we detected was that VEC-α-C associated more strongly to the actin cytoskeleton, as we showed by FRAP measurements and by Triton-X-100 extractability. Thus, we believe that the fusion between VE-cadherin and α-catenin stabilizes junctions by increasing the VE-cadherin association to the cytoskeleton.

It is generally accepted that VE-cadherin needs to form a complex with the catenins in order to establish stable cell junctions. The concept that the catenins link cadherins to the actin cytoskeleton has been challenged when the Nelson and Weis labs showed that α-catenin is an allosteric molecule that requires dimerization for binding to actin and that it is therefore incapable of binding to both β-catenin and actin filaments simultaneously.[28,29] However, it is still generally accepted that the cadherin-catenin complex is indirectly anchored to the actin cytoskeleton via linker molecules. These molecules can be, among other potential candidates, Eplin and also vinculin,[30,31] and both have been shown to interact with the VE-cadherin-catenin complex in endothelial cells.[32,33]

Vinculin has recently emerged as a modulator of cadherin strength, acting as a mechanotransducer linking the cadherin-catenin complex to the actin cytoskeleton under tension.[31] α-catenin can contribute to vinculin activation and also needs to overcome autoinhibitory interactions to coactivate vinculin.[34,35] Furthermore, it was shown that the vinculin binding site on α-catenin is usually masked and actomyosin mediated pulling forces are needed to unmask this site. This enables vinculin to bind and to strengthen its interaction with the actin cytoskeleton.[31] In endothelial cells, vinculin was found to localize to a distinct subset of VE-cadherin adhesions, defined as focal adherens junctions.[33] Agents compromising cell contact stability, for example VEGF, thrombin or TNFα, induced formation of these focal adherens junctions causing tension that pulled on the VE-cadherin-catenin complex. This tension increased vinculin recruitment to α-catenin at these sites of adhesion, a process that is needed for reinforcing the stability of adherens junctions. In agreement with this model it was recently reported that Eplin is necessary to recruit vinculin to adherens junctions.[32] Thus, Eplin could connect the VE-cadherin-catenin complex to the actin cytoskeleton which would allow this complex to sense tension, expose the masked vinculin binding site on α-catenin and thereby allow vinculin to reinforce stability of the junction.

Our finding that the VEC-α-C fusion protein strongly inhibits the opening of endothelial junctions in vivo in conjunction with its enhanced interaction with the actin cytoskeleton enforces the hypothesis that dissociation of the VE-cadherin-catenin complex from actin is needed to decrease its adhesive strength (**Fig. 3**). Indeed, several reports show the dissociation of catenins from VE-cadherin in response to various stimuli.[36,37] However, other reports show that the cadherin-catenin complex stays intact although the contacts are opened.[38-41] Thus, decrease of junctional strength during induction of permeability or leukocyte transmigration does not always lead to disassembly of the cadherin-catenin complex. This would suggest that VEC-α-C may not stabilize the VE-cadherin interaction with the actin cytoskeleton via preventing the dissociation of the VE-cadherin-catenin complex. Instead it is possible that due to the truncation of the N-terminal third of α-catenin in our VEC-α-C fusion protein, the binding site for vinculin might be constitutively accessible for vinculin and therefore enhance the interaction with actin in a constitutive way. Alternatively it is conceivable that the truncation of α-catenin changes its structure in a way

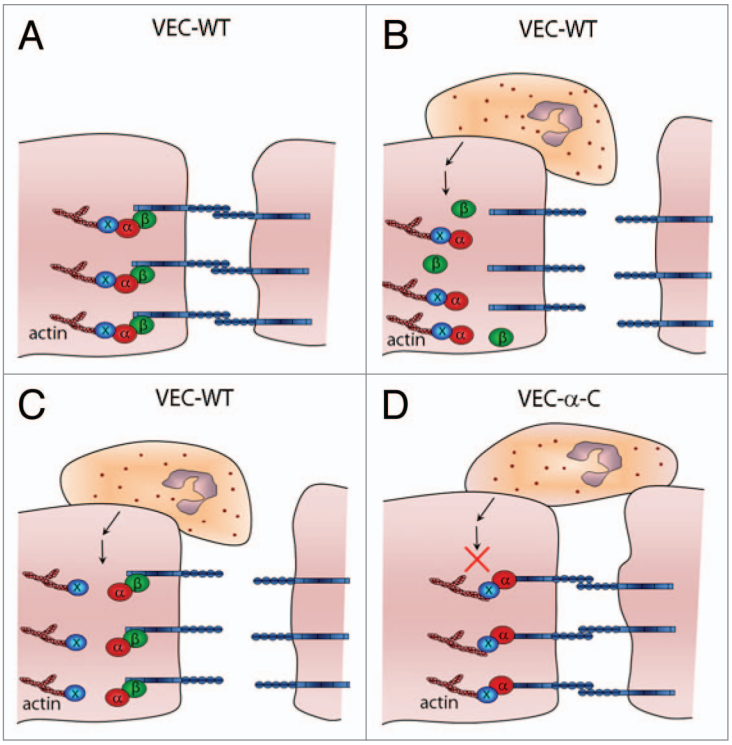

Figure 3. Two ways to dissociate the anchoring of VE-cadherin to the actin cytoskeleton and how VE-cadherin-α-catenin could interfere. (**A**) VE-cadherin is linked via β-catenin, α-catenin and bridging factors (X), such as possibly Eplin or Vinculin or α-actinin to the actin cytoskeleton. Leukocyte binding to endothelial cells or stimulation of permeability inducing factors could (**B**) dissociate the cadherin-catenin complex, or could (**C**) alternatively interfere with the bridging to the actin cytoskeleton. (**D**) The VE-cadherin-α-catenin fusion protein would for obvious reasons prevent the dissociation of the cadherin-catenin complex, and could possibly also block the dissociation from the bridging factor due to potential allosteric effects on the interaction between α-catenin and the bridging factor.

that enables it to bind actin although it is not dimerized.

Whatever the mechanism may be that stabilizes the interaction between the VEC-α-C fusion protein and the actin cytoskeleton, the outcome clearly is that in several tissues endothelial junctions are strongly reinforced leading to a complete block of vascular permeability induction in the skin and a strong inhibition of leukocyte extravasation in different tissues such as the cremaster, the skin and the lung. This is strong evidence for the importance of the junctional route for leukocyte extravasation in these tissues. The complete lack of inhibition of leukocyte extravasation in lymphocyte homing and the entry of neutrophils into the peritoneal cavity reveals the heterogeneity of the molecular mechanisms that control leukocyte diapedesis in different tissues. Future studies will be necessary to clarify whether leukocyte diapedesis in these tissues indeed occurs via different routes, e.g., transcellularly or whether the opening of endothelial junctions in lymph node HEVs and in postcapillary venules of the peritoneum is regulated differently.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflict of interest or financial interests.

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Abbreviations

DNFB, dinitrofluorbenzene; DTH, delayed type hypersensitivity; E-cadherin, epithelial cadherin; Eplin, epithelial protein lost in neoplasm; ESAM, endothelial cell-selective adhesion molecule; FRAP, fluorescence recovery after photobleaching; GPCR, G protein-coupled receptor; Gr-1, granulocyte differentiation antigen-1; HEV, high endothelial venule; HUVEC, human umbilical vein endothelial cells; ICAM-2, intercellular adhesion molecule-2; IL-1β, interleukin-1β; KO, knock-out; LPS, lipopolysaccharide; LSM, laser scanning microscope; LSP-1, lymphocyte-specific protein-1; MAdCAM-1, human mucosal addressin cell adhesion molecule-1; PBS, phosphate buffered saline; PECAM-1, platelet / endothelial cell adhesion molecule-1; PNAd, peripheral node addressin; TNF-α, tumor necrosis factor-α; VEC-α-C, VE-cadherin-α-catenin; VE-cadherin, vascular endothelial cadherin; VEC-WT, VE-cadherin wild type; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; VE-PTP, vascular endothelial protein tyrosine phosphatase

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