Endothelial Junction Regulation: A Prerequisite for Leukocytes Crossing the Vessel Wall

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Abstract

The leukocytes of the innate immune system, especially neutrophils and monocytes, exit the circulation early in the response to local inflammation and infection. This is necessary to control and prevent the spread of infections before an adaptive immune response can be raised. The endothelial cells and the intercellular junctions that connect them form a barrier that leukocytes need to pass in order to get to the site of inflammation. The junctions are tightly regulated which ensures that leukocytes only exit when and where they are needed. This regulation is disturbed in many chronic inflammatory diseases which are characterized by ongoing recruitment and interstitial accumulation of leukocytes. In this review, we summarize the molecular mechanisms that regulate endothelial cell-cell junctions and prevent or permit leukocyte transendothelial migration.

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Introduction

Endothelial cells are a specialized cell type that lines the inner lumen of all blood and lymph vessels. They are at the interface between blood and surrounding tissues. The cell-cell contacts between endothelial cells are highly regulated and determine permeability to cells (leukocytes, red blood cells), macromolecules (hormones, cytokines), nutrients (amino acids, sugars) and solutes (ions). They also form a barrier to blood-borne infectious agents.

Endothelia are heterogeneous both in function and structure, depending on the vessel type (arterial vs. venous), size (macrovasculature vs. microvasculature) and the organ of origin (e.g., central nervous system vs. lymphoid organs) [1]. On the one hand, the endothelial barrier can be very stringent. This is the case in the central nervous system where endothelial cells together with surrounding pericytes and glial cells form the blood-brain barrier. The blood-brain barrier limits the entry of immune cells and immune surveillance of the central nervous system and, at the same time, the entry of microorganisms and susceptibility to microbial infections [2]. On the other hand, the endothelial barrier can be very per-

Fig. 1. Leukocyte adhesion and transmigration cascade. Leukocytes from the blood stream roll on selectins expressed by endothelia at sites of inflammation (1). Activation through cytokines deposited on endothelial cells leads to activation of leukocyte integrins and firm adhesion to ICAM-1 or VCAM-1 (2). Adhered leukocytes then crawl along chemotactic gradients on endothelial cells (3). Interaction with

the endothelial cell, especially through ICAM-1 and VCAM-1, leads to the formation of docking structures (4) and signaling events that destabilize endothelial cell junctions and induce transmigration through endothelial cell junctions (paracellular) (5) or through the cell body (transcellular) (6). Transmigrated leukocytes can then crawl in the subendothelial space and enter the perivascular tissues (7).

missive to leukocytes, as is the case in high endothelial venules of secondary lymphoid organs where lymphocytes constitutively exit the blood stream [3]. These examples are extreme cases in a spectrum of endothelial barrier properties, and the majority of vessel types fall in between these two extremes.

Under physiological conditions, endothelia in the arterial branch of the vascular system are characterized by low permeability to solutes and leukocytes, compared to those in the venous branch where the endothelial cells are thinner and where leukocytes can exit the circulation more easily [4]. In response to local inflammation, the permeability to small and large molecules as well as to immune cells is greatly increased, particularly in postcapillary venules [1, 5]. The tight regulation of the endothelial cell barrier both under physiological and inflammatory conditions is crucial. It can have detrimental effects on human health when the regulatory mechanisms are disturbed. These range from exposure of subendothelial extracellular matrix and consequent thrombus formation to overshooting inflammation caused by the constitutive influx of leukocytes, as is the case in many autoimmune and other chronic inflammatory diseases such as rheumatoid arthritis [6].

Transendothelial Migration

A key event in the initiation of inflammation is the migration of immune cells out of blood vessels and into inflamed tissues. For this, leukocytes must cross a layer of

endothelial cells in a process called ‘transendothelial migration’, transmigration, diapedesis or extravasation. On their way out of blood vessels, leukocytes essentially take one of two routes: (1) the paracellular route between endothelial cells, i.e. through the adhesion molecules that form the endothelial cell-cell junctions, or (2) the transcellular route through the body of endothelial cells which leaves the junctions intact (fig. 1). The proportion of either of the routes taken varies depending on the type of endothelial cells (micro- vs. macrovascular) and leukocytes (monocytes, neutrophils, lymphocytes) used in experiments and on whether experiments were performed with endothelial cell lines or in vivo [for a review, see ref. 7, 8]. The initial belief was that most leukocytes use the paracellular route for transmigration. However, due to difficulties that arise from observing transcellular migration events, this type of migration may have been largely underestimated in earlier studies: for one, in human umbilical vein endothelial cells, the most commonly used endothelial cell line, transcellular migration, only made up 5–10% of transmigration events, whereas with other endothelial cell lines, up to 40% of leukocytes used the transcellular route [7]. Further, the majority of transcellular migration events occur in close proximity to endothelial cell junctions where they can easily be mistaken for paracellular migration when light microscopic methods are used. Only in painstakingly undertaken studies using serial-section electron microscopy was it possible to differentiate unambiguously between transcellular and paracellular transmigration at perijunctional sites [9–12].

The two modes of endothelial transmigration are non-redundant and may serve different functions, as blocking one pathway does not augment the other [13]. While the exact functions and benefits of either route are still under debate, Lossinsky and Shivers [14] hypothesize that the transcellular route may be preferred as the 'route of least resistance' in endothelial beds with extended intercellular junctional complexes, e.g., the microvascular endothelia of the blood-brain barrier. Transcellular migration leaves the intercellular junctions intact and ensures specific and regulated extravasation of leukocytes. For example, the transcellular route was used exclusively by leukocytes in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [15–17]. The exact regulatory mechanisms, the proportion and the function of this mode of transmigration remain to be clarified. In keeping with the scope of this review, we will go on to discuss the mechanisms underlying paracellular migration. The reader is referred to the following publications for a thorough review of transcellular transendothelial migration [7, 8].

The process of transendothelial migration is initiated by perivascular mast cells that produce proinflammatory mediators, like tumor necrosis factor- α (TNF- α) or interleukin-1 β , in response to an infection or tissue damage. Proinflammatory mediators induce signaling pathways in endothelial cells that cause an increase in vascular permeability for molecules and leukocytes [18]. Of equal importance, these mediators also trigger endothelial cells to express adhesion molecules, especially selectins and the integrin ligands intracellular cell adhesion molecule 1 (ICAM-1) and vascular endothelial cell adhesion molecule 1 (VCAM-1). Circulating leukocytes express integrins and other ligands for the recognition of and adhesion to inflamed endothelia. In a multistep process that includes a sequence of low- and high-affinity interactions of leukocytes with endothelial cells, followed by crawling on the luminal surface of vessels, leukocytes can ultimately migrate through the vessel wall (fig. 1) [19]. What ultimately determines where leukocytes transmigrate has not been conclusively clarified. It was suggested in an earlier study that neutrophils preferred tricellular corners where endothelial junctions are discontinuous [20]. In a recent study, it was shown that chemokines are released from endothelial vesicles at sites of contact with certain sets of effector T lymphocytes. These vesicles marked 'hot spots' where these T lymphocytes preferentially transmigrated [21]. Whether other types of leukocytes use similar chemokine hot spots remains to be clarified.

The first wave of immune cells that enter a site of inflammation are neutrophils, followed by monocytes and

later lymphocytes [22]. Together, the neutrophils and monocytes engulf microorganisms and damaged tissue by phagocytosis and keep infection in check until an antigen-specific adaptive immune response can be mounted. They also release cytokines that attract further leukocytes to the site of inflammation. The influx of new leukocytes from the bloodstream to sites of inflammation is one of the early check points in this process and therefore of particular importance. In many chronic inflammatory diseases, the endothelial barrier is disturbed and a large number of leukocytes can accumulate and damage the surrounding tissues. One such example is rheumatoid arthritis, an autoimmune disease that affects one or several joints. In rheumatoid arthritis, adhesion molecules, especially selectins, are upregulated on blood vessels of the synovial membrane of joints. This in combination with the local release of inflammatory chemokines such as CXCL5 or CCL2 leads to a massive influx of leukocytes into the synovial cleft and ultimately to severe damage to the joint [23].

In summary, transendothelial migration is a complex multistep process in which leukocytes need to overcome several barriers including the endothelial cell layer in order to get to sites of inflammation. In the following, we will describe the barriers imposed by endothelial cell-cell junctions and how they are regulated, especially during transmigration.

Endothelial Cell-Cell Junctions

Endothelial cell-cell junctions connect neighboring cells at their lateral borders. The junctions present one of the main obstacles that leukocytes encounter on their way out of blood vessels. They act as a gateway for transmigrating leukocytes and a primary point for the regulation of transmigration. On a molecular level, the junctions are formed by the multi-protein complexes of the lateral border recycling compartment (LBRC), tight junctions and adherens junctions (fig. 2).

Lateral Border Recycling Compartment

The LBRC is a specialized membranous organelle that is located in close vicinity to endothelial cell-cell junctions. Associated with it are the endothelial adhesion molecules platelet/endothelial cell adhesion molecule 1 (PECAM-1) and CD99 which form homophilic adhesions between endothelial cells [24]. Through the LBRC, PECAM-1 and CD99 are transported to either the apical or lateral cell surface where they are available for interac-

Fig. 2. The three multiprotein complexes that form endothelial cell-cell junctions (not drawn to scale). (1) Endothelial adherens junctions consisting of VE-cadherin which associates with intracellular p120-catenin and β -catenin (β -cat) and protein tyrosine phosphatases, like SHP2, and kinases (not shown). (2) Tight junctions are composed of occludin, claudins (claudin-5 is endothelial specific but other claudins are also expressed) and JAM-A, JAM-B and JAM-C. On the cytoplasmic side, tight junction proteins are associated with proteins like ZO-1 and indirectly with the cytoskeleton. (3) The lateral border recycling compartment with PECAM-1 and CD99 (JAM-A can also be found in the lateral border recycling compartment) underlies the adjoining cell membranes and transports the proteins from the abluminal to the apical and lateral sides where the proteins can interact with proteins on adjacent cells or form contacts with transmigrating cells.

tions with their homophilic ligands on leukocytes. PECAM-1 and CD99 contribute to trans- and paracellular modes of migration, respectively [25]. Furthermore, when endothelial PECAM-1 is blocked, leukocytes can adhere and crawl on the luminal surface of blood vessels but cannot transmigrate [26]. When CD99 is blocked, leukocytes can start to move through the endothelial junctions but then ‘get stuck’ and cannot complete transmigration [27, 28], suggesting that the two proteins act in sequence to expedite diapedesis. The role of the LBRC during transmigration is to shuttle PECAM-1 and CD99 back to the apical side of endothelial cells where they are then available for further transmigration events. This makes the LBRC and the proteins associated with it indispensable for efficient transendothelial migration.

Endothelial Tight Junctions

Tight junctions form belts of adhesion molecules at the lateral border of endothelial cells where their main function is to control paracellular diffusion of small molecules [2]. Endothelial tight junctions are composed of the integral transmembrane proteins occludin, members of the claudin family, particularly the endothelium-specific claudin-5 [29], endothelial cell-selective adhesion molecule (ESAM) [30] and the junctional adhesion molecules (JAMs) A, B and C [31]. The variations in the barrier properties of endothelia in different vascular beds corre-

late with the abundance and complexity of endothelial tight junctions [32]: in transmission electron microscopy, the tight junctions of postcapillary venules present as punctate, electron-dense regions whereas the junctions of arterial endothelia and the blood-brain barrier are more extended and show high expression of occludin [4, 33, 34]. In the brain these observations coincide with the immune surveillance being 100 times lower compared to that in the spleen or in lungs [35].

ESAM molecules form homophilic contacts between endothelial cells. Of interest, a role for ESAM in opening endothelial junctions was inferred from studies showing that neutrophil transmigration was reduced in ESAM^{-/-} mice [36, 37]. Moreover, monocyte adhesion and migration into atherosclerotic plaques was impaired in mice deficient for apolipoprotein E and ESAM [38]. It was suggested that ESAM contributed to the loosening of junctions by forming a platform for molecules that recruit and activate RhoA [39]. As we describe below, RhoA activation in endothelial cells can lead to the disruption of endothelial cell junctions, and thus, facilitate leukocyte extravasation. It remains unclear whether leukocytes can directly target ESAM-dependent junctions and destabilize them in order to transmigrate.

JAMs, as a part of tight junctions, mediate hetero- and homophilic interactions with JAMs on neighboring cells. JAMs are expressed on epithelial and endothelial cells in

many different organs [40] and are important for the formation of tight junctions and the establishment of the vascular diffusion barrier. Furthermore, JAM-A, JAM-B and JAM-C can bind to the leukocyte integrins LFA-1, VLA-4 and Mac-1, respectively [41–43]. JAMs, especially JAM-A which can also be found in the LBRC [25], are important for transendothelial migration of leukocytes and are upregulated in many inflammatory conditions [40]. The importance of JAM-A for transmigration is underlined by experiments that showed when JAM-A was blocked with monoclonal antibodies, transendothelial migration was decreased [41]. Further, a modified soluble form of JAM-A, which interferes with JAM-A LFA-1 interactions but does not affect homophilic JAM-A interactions, inhibits leukocyte recruitment to sites of inflammation [44].

Chavakis et al. [42] performed experiments in which injection of soluble JAM-C prior to intraperitoneal injection of thioglycollate significantly decreased the number of neutrophils in peritoneal lavage fluid. The authors of this study attributed the reduction in extravasated leukocytes to JAM-C being necessary for luminal to abluminal migration. An alternative explanation was offered by Imhof and colleagues [45] who used live-cell imaging to track monocytes during transmigration on TNF- α -stimulated human umbilical vein endothelial cell monolayers. These studies revealed that after the addition of soluble JAM-C, the number of transmigrated monocytes was initially the same as in control conditions, but that about 25% of the transmigrated monocytes moved back to the luminal side of the endothelial monolayer [45]. These findings were further corroborated by an *in vivo* study in which either JAM-C blocking antibodies or lowered JAM-C expression as a consequence of ischemia/reperfusion result in increased reverse transmigration of neutrophils from the abluminal to the luminal side of vessels. Overall, total number of transmigrated neutrophils compared to controls was reduced in those experiments [46]. This indicates that JAM-C ensures unidirectional migration possibly by acting as a ‘valve’ that permits the transmigration out of vessels but restricts the natural propensity of leukocytes for reverse transmigration. This mechanism may be of particular importance to ensure polarized migration into lymph nodes in high endothelial venules, where leukocytes constitutively exit the circulation and where JAM-C is highly expressed [47].

In summary, tight junctions have an important role for the maintenance of junctional integrity and the permeability barrier formed by endothelial cell-cell junctions. Independent of their roles in the tight junctional

complex, some components of tight junctions like ESAM and JAMs play an active role in promoting leukocyte diapedesis.

Endothelial Adherens Junctions

Adherens junctions, like tight junctions, mediate endothelial cell-cell adhesion. Endothelial adherens junctions are formed by vascular endothelial (VE) cadherin which regulates paracellular permeability to macromolecules [48, 49]. Further, the importance of VE-cadherin for transendothelial migration was demonstrated in mice: intravenous administration of antibodies that block VE-cadherin homophilic interactions lead to increased vascular permeability in the heart and lung and to the accumulation of neutrophils in inflamed peritoneum [50, 51].

Structurally, VE-cadherin is a classical cadherin with an extracellular domain that mediates Ca²⁺-dependent homophilic adhesion, a transmembrane region and a conserved cytoplasmic tail. The cytoplasmic domain interacts directly with p120-catenin and β - or γ -catenin, a reciprocally exclusive interaction. VE-cadherin is also associated with α -catenin, the cytoskeleton and other signaling molecules [52]. The formation of the cadherin-catenin complex is necessary for VE-cadherin expression on the cell surface. Especially the interaction with p120-catenin prevents internalization and degradation of VE-cadherin [53], and silencing of p120-catenin leads to decreased expression of VE-cadherin and α -, β - and γ -catenin and a drop in transendothelial electrical resistance [54]. Similarly, in epithelial cells, β -catenin ensures the expression of E-cadherin at the plasma membrane by preventing the ubiquitination and degradation of E-cadherin [55]. However, the long-standing assumption that β -catenin directly links E- and VE-cadherin to α -catenin and thus to the actin cytoskeleton has come under debate since the labs of Nelson and colleagues [56, 57] demonstrated in biochemical experiments that α -catenin cannot bind to β -catenin and actin simultaneously. Even though the role of α -catenin in this context has not been conclusively clarified, there is ample evidence for the connection of cadherins with the actin cytoskeleton through other actin-binding molecules [58]. The presence of α -catenin in the cadherin-catenin complex is nonetheless integral to the stability and function of VE-cadherin-mediated adherens junctions. This is underlined by findings from Schulte et al. [13] who designed a chimeric protein comprising the extracellular and transmembrane parts of VE-cadherin fused to the C-terminal part of α -catenin. Expression of this chimera prevents the vascular endothelial growth factor (VEGF)- or histamine-induced perme-

ability increase in mouse skin. Further, leukocyte adhesion remained unaltered, whereas transmigration was blocked and leukocyte recruitment to inflamed lung or skin tissues was greatly decreased.

Taken together, VE-cadherin and the associated catenins are of crucial importance to the formation and stability of adherens junctions, and thus, to the regulation of leukocyte transmigration. In the following, we will describe mechanisms by which endothelial cells modify junction proteins and therefore allow or restrict leukocyte movements across endothelia.

Regulation of Endothelial Junctions

Endothelial junctions are tightly regulated which ensures adequate barrier function under physiological conditions. The junctions are also amenable to rapid adjustments during inflammation without disrupting the gross integrity of endothelial cell layers but allowing the influx of immune cells. Inflammatory stimuli like TNF- α , interleukin-1 β or interferon- γ , but also engagement of integrin ligands, increase endothelial permeability to molecules and leukocytes. This requires signaling pathways that trigger the remodeling of endothelial cell-cell junctions and enable leukocyte extravasation.

Metalloproteinases

A mechanism that is often omitted from reviews on transendothelial migration is the exodomain shedding of adhesion molecules that form the endothelial barrier, particularly VE-cadherin, by matrix metalloproteinases (MMPs) or ADAMs (a disintegrin and metalloproteinase). This is surprising considering that elevated levels of soluble VE-cadherin, corresponding to a 90-kDa extracellular fragment of VE-cadherin, correlate with disease activity of rheumatoid arthritis, diabetic retinopathy and coronary atherosclerosis, all conditions characterized by chronic infiltration with inflammatory cells [59–61]. Both types of enzymes are produced by leukocytes and endothelial cells alike. Of the MMPs, in particular MMP-2 and MMP-7 have been shown to cleave the extracellular domain of VE-cadherin, e.g., in response to TNF- α [22, 61, 62]. However, a direct connection between VE-cadherin shedding by MMPs and leukocyte transmigration has not yet been shown.

Like MMPs, the enzymes of the ADAM family are also upregulated in response to inflammatory mediators. Several lines of evidence suggest that ADAM-mediated VE-cadherin shedding may facilitate leukocyte transmigration.

Pharmacological inhibition of ADAM10 reduces VE-cadherin shedding and monocyte migration [63]. By a similar mechanism, the inhibition or knockdown of ADAM10 in either endothelial cells or leukocytes blocks the transmigration of T lymphocytes [64], and finally, knockdown of ADAM15 in endothelial cells reduces neutrophil migration [65, 66]. These results demonstrate that in the absence of ADAM activity, the number of migrating leukocytes is reduced, whereas when ADAM expression is upregulated in response to inflammatory mediators, one may speculate that diapedesis would be promoted. It is interesting to note that both ADAM10 and ADAM17 can shed JAM-A, which results in an overall decrease in leukocyte transmigration. This may be due to the shed JAM-A exodomains blocking the binding sites for LFA-1 on leukocytes [67]. The role of ADAMs in vascular biology is more extensively reviewed in Dreymueller et al. [68].

In summary, especially the ADAM-type metalloproteinases affect transmigration. By shedding VE-cadherin exodomains, ADAMs can open the cell-cell junctions for diapedesis, whereas by shedding JAM-A exodomains, endothelium-leukocyte interactions are blocked and diapedesis inhibited. The exact regulation and coordination of these events requires further study and many questions remain unanswered. It will be interesting to know how exodomain shedding affects the stability of the cadherin-catenin complex. More important still is the question whether the VE-cadherin exodomain shedding that can be observed in some inflammatory diseases occurs as a prerequisite for or as a consequence of transmigration. In the former case, metalloproteinases could be essential for the initiation, and in the latter case, for the perpetuation of an inflammatory infiltrate into tissues. Metalloproteinase inhibitors are already used in the therapy of cancer but may also be valuable new targets for therapeutic intervention in inflammatory diseases.

Protein Tyrosine Kinases and Phosphatases

Tyrosine phosphorylation is a mechanism often employed to bring about rapid changes in protein interactions. Of the proteins in endothelial cell-cell junctions, VE-cadherin especially is a target for tyrosine phosphorylation [69, 70]. There are nine tyrosine residues in the cytoplasmic tail of VE-cadherin. In particular, phosphorylation of tyrosine residues Y658 and Y731 is associated with increased endothelial permeability and facilitates transendothelial migration. When these residues are phosphorylated, p120- and β -catenin, respectively, can no longer interact with VE-cadherin, which leads to the

dissociation and internalization of the cadherin-catenin complex [71]. Conversely, overexpression and binding of p120-catenin to VE-cadherin contributes to junctional stability by preventing the phosphorylation of VE-cadherin on Y658 and, at the same time, inhibits diapedesis of neutrophils [72, 73].

Among the stimuli that induce tyrosine phosphorylation in endothelial cells are histamine, thrombin, platelet-activating factor, VEGF and TNF- α [74–76]. Similarly, ICAM-1 crosslinking with antibody-coated beads or integrin-mediated leukocyte adhesion lead to the recruitment and activation of the tyrosine kinases Src and Pyk2 at sites of adhesion and concomitant phosphorylation of VE-cadherin [77–79]. In the same studies, leukocyte transmigration was prevented when non-phosphorylatable VE-cadherin mutants were expressed in endothelial cells [77, 79]. These findings indicate that tyrosine phosphorylation of endothelial junction proteins is indispensable for effective leukocyte transmigration. There have been some reports on serine and threonine phosphorylation in response to VEGF which lead to the internalization of VE-cadherin and increased endothelial permeability [80]. Whether serine/threonine phosphorylation events are also employed to facilitate leukocyte transmigration is not yet clear.

In a recent study, in which both *in vivo* and *in vitro* endothelial cell systems were used, the low shear stress typical for venous vessels was identified as a regulator of VE-cadherin phosphorylation status [81]: in veins, VE-cadherin is constitutively phosphorylated on tyrosine residues Y658 and Y685, whereas in arterial-type vessels, VE-cadherin remains largely unphosphorylated on those residues. Further, application of arterial flow to veins, by introducing a shunt from the carotid artery to the jugular vein, decreased the phosphorylation of Y658 and Y685. The authors show that venous shear stress activates Src-mediated VE-cadherin phosphorylation which leaves the endothelial barrier function intact but makes the endothelial cells more susceptible for rapid opening of junctions in response to inflammatory mediators.

The actions of protein kinases are held in check by protein tyrosine phosphatases (PTPs). Phosphatases counteract the action of tyrosine kinases and may thus stabilize junctions by dephosphorylating VE-cadherin and the catenins. In endothelial cells, density-enhanced phosphatase 1, VE receptor-type PTP, PTP μ , PTP1B and Src-homology 2 domain containing phosphatase 2 (SHP2) have been described. The best-studied example is VE-PTP which localizes to the junctions of confluent endothelial cells and dissociates from VE-cadherin in response to

VEGF or when neutrophils or T lymphocytes extravasate [82, 83]. More recently, SHP2 has been described to stabilize VE-cadherin in response to endothelial fibroblast growth factor [84] and to aid the recovery of VE-cadherin-mediated junctions after thrombin treatment [85]. More research is required to clarify if SHP2 inhibition could facilitate transmigration. Overall, the balance of phosphorylation and dephosphorylation of VE-cadherin determines the stability of endothelial cell junctions and whether or not leukocytes can transmigrate.

Shear Stress

Endothelial cells experience shear stress caused by blood flow. Shear stress can be sensed and translated into intracellular signals by mechanosensing complexes, including ion channels, the glycocalyx and a mechanosensory complex consisting of VEGF receptor 2 and the junctional proteins VE-cadherin and PECAM-1 [86, 87]. Shear stress is both an impediment and a necessity for efficient transmigration. On the one hand leukocytes in the circulation need to slow down sufficiently before they can cross the vessel wall. For this, they engage in low-affinity adhesive interactions with selectins that cause rolling adhesion of leukocytes on the endothelium (fig. 1) [88]. On the other hand selectin-mediated adhesion requires at least low levels of shear stress for efficient binding to selectin ligands [89]. Shear stress also enhances transendothelial migration of T lymphocytes on endothelia coated with chemokines, whereas chemokines alone only support luminal crawling [90]. Another way in which shear stress affects leukocyte transmigration is by altering gene expression. Especially, disturbed or turbulent flow can induce the expression of inflammatory genes including the adhesion molecules ICAM-1 and VCAM-1 [91]. Vascular inflammation in response to disturbed flow at vascular bifurcations has been studied extensively in the context of atherosclerosis which is characterized by the accumulation of macrophages in the vessel wall [92].

While there is no doubt that shear stress is necessary for efficient transcellular migration, to date, there have been no studies that directly correlate junctional stability and transmigration, on the one hand, with shear stress on the other hand. Nonetheless, there are several studies that implicate disturbed flow and low shear stress with increased junctional permeability and instability, and vice versa. As we have discussed above, low levels of shear stress lead to increased phosphorylation of VE-cadherin and more leakage through junctions [81]. *In vivo*, and *in vitro* when endothelial cells are subjected to high laminar

shear stress, the cells align in the direction of flow [87, 93, 94]. VE-cadherin-based junctions that lie in parallel to the direction of flow are linear and, presumably, more stable compared to the junctions that are perpendicular to flow and have a 'jagged' phenotype [93, 94]. The latter is reminiscent of the less stable focal adherens junctions in which vinculin, another mechanosensor, colocalizes with VE-cadherin [95]. With the new methods for intravital imaging, it will be possible to investigate whether transendothelial migration happens preferentially at the jagged junctions or at junctions that are phosphorylated and may thus provide the missing link between shear stress, junctional stability and transmigration.

Small GTPases

The actin cytoskeleton is an important mediator of endothelial cell-cell junctions. Small GTPases, as key regulators of the actin cytoskeleton, are involved in the formation and maintenance of cell-cell junctions [96]. The Rho family GTPases RhoA and Rac1 and the Ras family GTPase Rap1 have been shown to control endothelial junction stability and will be discussed in the following.

The activity of Rho GTPases is regulated by guanine-nucleotide exchange factors (GEFs) that enhance the exchange of GDP for GTP. The GTP-bound active form of the GTPases interacts with effector molecules which regulates their activity and localization within the cell. The signaling by Rho GTPases is attenuated by GTPase-activating proteins, which catalyze the intrinsic GTP hydrolyzing activity of the GTPases and transfers them to the GDP-bound inactive state [97]. GEFs and GTPase-activating proteins not only affect the activation state, but also the localization and timing of GTPase activity. Overall, an intricate balance of GEF-induced GTPase activation and GAP-mediated deactivation needs to be maintained to ensure that cells function properly.

The function of the Rho GTPases RhoA and Rac1 in epithelial cells has been studied in great detail. They play an important role in regulating E-cadherin-mediated junction formation and maintenance [98]. Yamada and Nelson [99] have described that during initial cell-cell contact formation, newly forming E-cadherin-based adhesion sites are surrounded by areas of localized Rac1 activation that help expand the contact zones. Simultaneously, local activation of RhoA was observed adjacent to but not overlapping with areas where Rac1 was activated. These specific, localized activities are in line with studies suggesting that Rac1 and RhoA are inversely regulated at junctions: recently, Yap and colleagues [100] showed that the Rho-GEF ECT2 binds via α -catenin to E-cadherin,

which locally activates RhoA, while the concomitant recruitment of MgcRacGAP inactivates Rac1. Vice versa, when Rac1 is activated upon cadherin ligation, cadherin-associated p120-catenin recruits p190RhoGAP which leads to local deactivation of RhoA and overall adherens junction stabilization [101].

In response to inflammatory stimuli, Rac1 contributes to barrier disruption in endothelial cells: for example, several groups have shown that TNF- α stimulation or VCAM-1 crosslinking on endothelial cells activates Rac1 and induces the generation of reactive oxygen species. Reactive oxygen species in turn inhibit tyrosine phosphatases which results in a net increase in tyrosine phosphorylation of junctional proteins, the destabilization of cell-cell junctions and an overall increase in endothelial permeability [102–106]. Further, activation of RhoA and its effector Rho kinase through hypoxia, thrombin or histamine induce the formation of stress fibers, retraction of cell borders and consequently gap formation between cells, junctional destabilization, dispersion of VE-cadherin contacts and a loss of endothelial barrier function [96, 107–109].

During transendothelial migration, endothelial Rho GTPases translate signals from clustered ICAM-1 into intracellular signals. Initially, RhoA is rapidly activated upon ICAM-1 clustering and mediates the formation of F-actin stress fibers and endothelial barrier permeability [110–115]. The small GTPases Rac1 and RhoG are activated at a later stage and control the formation of endothelial docking structures that are involved in leukocyte transmigration [115]. Blocking any one of the three GTPase, RhoA, RhoG or Rac1, perturbs leukocyte passage across the endothelial monolayer. However, whether the reverse is true that GTPases mediate junctional destabilization in order to allow leukocyte passage remains elusive and requires further study.

Finally, the Ras family small GTPase Rap1 plays a role in stabilizing VE-cadherin at junctions. Rap1 activation causes the tightening of adherens junctions [116–118]. Furthermore, the cAMP-Epac1-Rap1 pathway counteracts the thrombin-induced permeability increase by inhibiting RhoA activation [118–121]. On the one hand, inhibition of Rap1-mediated signaling by overexpression of Rap1-GAP or knockdown of Rap1 or its activating GEF, PDZ-GEF, leads to decreased barrier function [117, 122]. Interestingly, Orlova et al. [123] showed that induction of endothelial permeability through VEGF or histamine lead to a recruitment of the tight junction protein JAM-C from the cytosol to cellular junctions where it negatively regulates Rap1 functions. On the other hand,

JAM-C knockdown improves barrier function by decreasing stress fiber formation and phosphorylation of myosin light chains as well as by activation of Rap1. Conversely, JAM-A activates Rap1 and promotes adherens junction integrity [124, 125]. In line with these findings, pharmacological activation of Rap1 inhibited transmigration of leukocytes, whereas Rap1 inactivation by overexpression of Rap1 GAP had the opposite effect [126].

As we have described above, there is compelling evidence that GTPase activity regulates essential steps of leukocyte transendothelial migration by inducing actin remodeling and by regulating junction stability and the formation of docking structures that are necessary to assist leukocytes on their route through the vessel wall.

Concluding Remarks

We have discussed how endothelial cells regulate cell-cell junctions in the steady state and during leukocyte transmigration. As a final note, we want to re-emphasize that transmigration is a highly regulated event that requires the active participation of both the transmigrating

leukocytes and endothelial cells. It is important to realize that although increased permeability and transmigration often occur at the same time, e.g., in response to an inflammatory stimulus, and share some of the same signaling pathways, they are two distinct events. 'Opening' VE-cadherin-mediated junctions with blocking antibodies leads to increased endothelial permeability and leukocyte transmigration [51]. However, when firm adhesion and signaling through ICAM-1 is additionally blocked, fewer leukocytes can transmigrate [127]. This suggests that, even in the absence of flow, leukocytes cannot just 'fall through' open junctions and that the endothelium has functions that go beyond just forming a barrier to leukocyte transmigration. Instead, the endothelial lining of vessels actively enables transmigration, not only by forming a substrate for leukocyte adhesion and crawling but also by providing directional cues in the form of chemokines, by inducing the activation of integrins on leukocytes and by ultimately opening the junctions to transmigrating leukocytes when and where it is necessary. Only through the extensive communication between leukocytes and endothelial cells can the endothelium ensure the regulated and targeted extravasation of specific immune cells.

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