Chapter 2

Cell-matrix adhesion proteins in the regulation of endothelial permeability

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CONTENT

1 – INTRODUCTION

1.1. Endothelial barrier function and regulation

Throughout the body, the vasculature is lined with a thin, continuous sheet of endothelial cells. This monolayer of tightly adhering cells regulates – in addition to a broad spectrum of other processes – the barrier properties of the vascular wall. The endothelial barrier allows extravasation of water, electrolytes, proteins, monocytes and leukocytes to supply the surrounding tissues. The balance between barrier function and regulated extravasation results from stringently controlled permeability of the endothelial monolayer, which prevents excessive vascular leak and interstitial edema. Endothelial permeability is regulated at various levels, and is determined by environmental conditions, such as inflammation and hypoxia, and the location of the endothelium in the body.

Plasma constituents pass the endothelial barrier via two pathways, the transcellular and the paracellular pathway. Transcellular exchange predominates under basal conditions – hormones and macromolecules are transported from the luminal to the abluminal site of the endothelium via a system of caveola or caveolar channels in most types of continuous endothelia. During paracellular transport plasma constituents pass through gaps between neighbouring endothelial cells. This type of exchange is limited under basal conditions, but after exposure to vasoactive agents (like histamine), leukocyte-derived cytokines or angiogenic factors (like vascular endothelial growth factor [VEGF]), the interendothelial cell contacts are loosened permitting local or even extensive leakage of fluid and proteins into the interstitium. Disruption of cell-cell contacts and the consequent edema formation characterizes inflammatory conditions like sepsis and acute lung injury [1]. Transport of macromolecules via the transcellular pathway takes places at a continuous, low rate, while the paracellular route is more dynamic, and subject to various and complex regulatory mechanisms. The paracellular pathway should not only allow passage of larger proteins and leukocytes, requiring large plasticity of the intercellular adhesions, but is also subject to continuous remodeling of cell-cell contacts, even in a resting endothelial monolayer [2]. Despite high plasticity of cell-cell contacts and large differences in oncotic or hydrostatic pressure, the endothelium effectively seals the vessels and prevents leakage of fluid to the interstitium. This endothelial barrier is the resultant of three major forces: actomyosin contractile force, tethering forces in cell-cell contacts (or junctions) and the adhesive force of the endothelial cell to the extracellular matrix (Figure 1). In a resting monolayer the tethering forces in the cell-cell contacts balance the relatively low forces resulting from actomysion contraction. Adhesion of endothelial cells to the extracellular matrix (ECM), in short cell-matrix interaction, is the third factor, which contributes to the spread and flat shape of endothelial cells in a resting monolayer.

The current chapter specifically focuses on the contribution of cell-matrix interaction to the regulation of endothelial barrier function. Before discussing the modulation of endothelial permeability by cell-matrix interaction in detail, we shall briefly indicate how endothelial cell-cell junctions and the actin cytoskeleton determine the endothelial barrier function. For an excellent overview of endothelial barrier regulation in general we refer to the review of Mehta and Malik [2].

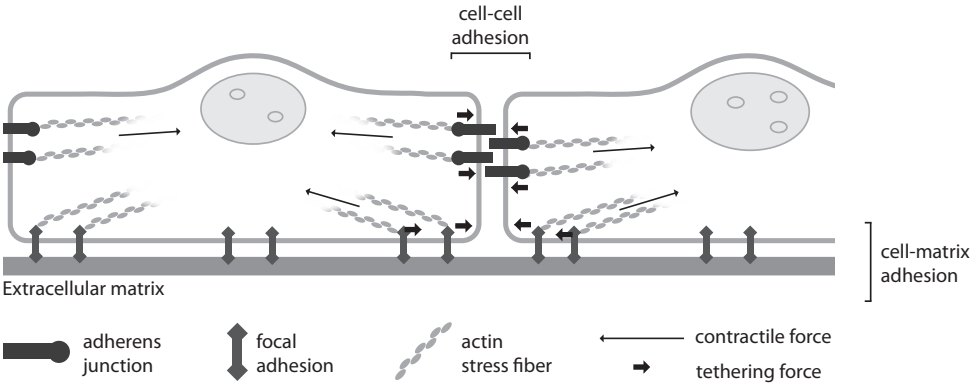

Figure 1 Overview of forces observed in endothelial cells within a resting endothelial monolayer. Actin fibers exert contractile forces by actomyosin interaction. These centripetal forces are balanced by tethering forces in cell-cell adhesions, predominantly the adherens junctions, and in the cell-matrix adhesion sites (focal adhesions). Under basal condition these forces are in balance, resulting in maintenance of spread cell shape.

1.2 Endothelial junctions

The endothelial monolayer is sealed by junctional structures, which limit the exchange of plasma solutes and circulating cells. Only in pathophysiological conditions, such as exposure to vasoactive agents, tumors and chronically inflamed tissues, the junctions (in particular in postcapillary venules) widen or dissociate, by which plasma macromolecules come abundantly available to the interstitial tissue. In endothelial cells three types of junctions are found: adherens junctions (AJs), tight junctions and gap junctions. Similar as in epithelial cells the barrier properties of endothelia are governed by adherens and tight junctions, while gap junctions contribute to intercellular communication. However, in most endothelia AJs are the major determinant of barrier function, and the molecular players in these junctions are specific and specifically regulated.

When a new junction is formed between two adjacent endothelial cells, exploratory lamellipodial protrusions form the initial contact between these cells [3,4]. As shown in cultured endothelial cells, these initial contacts contain a branched actin network with the Vasodilator-Stimulated Phosphoprotein (VASP) at their leading end [4]. Subsequent reorganization causes the retraction of the lamellipodia, accompanied by the formation of interdigitating filopodia-like structures,

which accumulate VE-cadherin and maintain the intercellular connection by homophilic VE-cadherin interactions. In this initial stage VE-cadherin displays a staining pattern that is perpendicular on the cell perimeter, a pattern that is also observed when vasoactive agents have destabilized the junction. Similar as E-cadherin in epithelial cells, the endothelial specific VE-cadherin is a dominant AJ protein in most endothelia. Homophilic VE-cadherin binding triggers interaction with catenins, which in turn facilitates indirect binding to the F-actin cytoskeleton. This is accompanied by a subsequent rearrangement of the cortical F-actin-myosin cytoskeleton along the cell perimeter, eventually resulting in a belt of AJs that seals individual endothelial cells together in a monolayer. Indeed, once stable junctions are formed, VE-cadherin is recognized as a thin lining between adjacent endothelial cells. Additional tight junctions enforce this belt. Only in specialized endothelia such as in the blood brain barrier, the retina and the testis, a sealing belt of tight junctions is formed like in epithelia. In most continuous endothelia of the body the tight junctions form interrupted mosaic-like structures that can be circumvented and passed by macromolecules [5], while the continuously present AJs act as the major determinants of permeability resistance in the junctions of these endothelia [6,7]. As a consequence AJs play a dominant role in the tethering forces of most types of endothelial cells [8].

Both AJs and tight junctions are coupled via regulatory adaptor proteins to the F-actin cytoskeleton (Figure 1). VE-cadherin interacts in particular with p120-catenin and β-catenin, which binds indirectly to F-actin. The stability of the homotypic VE-cadherin interaction is affected by specific phosphorylations of these proteins [9-11]. α-Catenin is generally thought to act as a connector between β-catenin and the F-actin fibers, although this interaction has been debated [12. Details on the regulation of the complex of VE-cadherin and catenins and its importance for endothelial permeability are given in several recent reviews [2,13,14].

1.3 The actin cytoskeleton

Actin is the most abundant intracellular protein, in particular in the cortical region of the endothelial cell, and amounts with myosin for about 16% of the intracellular protein mass. While part of the actin molecules is present as soluble, inactive G-actin monomers (globular form), they become easily liberated into an F-form that can multimerize into F-actin fibers. The F-actin fibers form thin actin networks, such as the branched F-actin networks in lamellipodia and the more parallel oriented F-actin fibers in filopodia. Most prominent are the thick, discrete structures generated by assembled F-actin fibers (e.g. stress fibers in migrating and activated endothelial cells) and the cortical F-actin rim at the cell margins of confluent cells. F-actin fibers often form contractile actin-myosin structures with non-muscle myosin. Actin-myosin structures play an important role in cell shape, tensile forces, cell migration and cell movement in general. Their generation and

modification is controlled by many structural and signaling molecules, amongst which the Rho GTPases Rac1, Cdc42 and RhoA play important and individually distinct roles.

The attachment of the actin cytoskeleton depends on the condition of the monolayer. In an intact endothelial monolayer, the cortical F-actin network interacts with the cell-cell junctions and condenses in a thin, pericellular F-actin lining. The exact dynamics of this interaction remains to be unravelled. At sites where endothelial cells loose their cell-cell contacts, F-actin fibers become attached to cell-matrix adhesion points, the presence of which rapidly increases during loss of cell-cell contact. In the front of a migrating endothelial monolayer (induced after removing several rows of cells by scratching) and in subconfluent endothelial cells the emerging focal adhesions are the nucleation points for the formation of stress fibers and subsequently provide – in addition to contributing to cell shape and tension - a signaling hub enforcing communication between cell and underlying matrix. When endothelial cell monolayers become suddenly exposed to shear forces a relatively thick type of stress fibers is also formed in the basolateral part of the cell [15], probably required for firm counteraction of the new forces on the cell. A new feature directly related to junction (de)stabilization is the formation of so-called radial F-actin stress fibers. Recent data show that endothelial cells after exposure to a barrier destabilizing stimulus often acquire this additional type of stress fibers that is connected to discontinuous AJs [16], and – when it occurs in both cells – connects two adjacent cells [17]. Using elegant laser ablation techniques, Huveneers et al. showed that these radial F-actin bundles exert pulling forces on AJs [16]. As we shall discuss below, it is plausible that during the destabilization of AJs vinculin interacts with α-catenin and forms a new interaction site for newly forming stress fibers, which as a safety net may contribute to limiting paracellular permeability and/or restoring the original intact junctional organization.

The structure and regulation of F-actin filaments is tightly linked to the other main cytoskeletal component, the microtubules. Microtubules are multimeres of the structural protein β-tubulin, and are together with microfilaments responsible for cytoskeletal maintenance of cell shape [18]. Therefore, microtubule disassembly, as observed during endothelial stimulation with vasoactive agents, contributes to disruption of the endothelial barrier [19,20].

1.4. Endothelial Cell-ECM interaction

Compared to cell-cell junctions and actomyosin contraction, the involvement of cell-matrix interaction in endothelial barrier regulation is less well-defined. Cell-matrix interaction is organized and regulated at four different levels: 1) the composition of the extracellular matrix, 2) the integrins, a large family of dimeric, transmembrane adhesion receptors, 3) the actin cytoskeleton, and 4) the focal adhesion (FA), an intracellular, multi-protein signaling complex that

connects the integrin receptors to the cytoskeleton (Figure 2). The poor understanding of the role of cell-matrix interaction in endothelial barrier regulation has several explanations, including the complex regulation at the various levels, the multidirectional character of inside-out and outside-in signaling, the methodological difficulty to study cell-matrix interaction independent of actomyosin contraction, and the often embryonically lethal phenotype of knockout mice (due to vascular instability) [21]. Understanding the involvement of cell-matrix interaction in endothelial barrier regulation has direct clinical relevance. It may provide additional targets to intervene in endothelial barrier disruption, and contribute to treatment of vascular leak and edema formation. It also yields valuable information on drugs that affect cell-matrix interaction.

In this chapter we provide an overview of proteins involved in cell-matrix interaction and their role in endothelial barrier regulation. The first part of the chapter discusses endothelial barrier regulation at the different levels of cell matrix interaction. Subsequently, we will discuss the regulation of cell-matrix interaction in the context of other barrier-regulating process, in particular cell-cell junction dynamics. Integrating existing literature, we will discuss a model in which both cell-cell junctions and FAs exert tethering forces to balance the contractile forces of the actin cytoskeleton. The chapter closes with concluding remarks and clinical implications.

2 – Cell-matrix interaction and endothelial barrier regulation

2.1. The role of the extracellular matrix

Adhesion to extracellular matrix (ECM) is essential for endothelial cells to survive, to proliferate and to exert their normal function. In the blood vessels, this ECM is provided by the basement membrane in mature vessels, or by the interstitial or temporary fibrin matrix during sprouting angiogenesis. The ECM consists of a fine meshwork of filamentous proteins, the composition of which is different between an mature basement membrane and the interstitial or wound matrix through which angiogenic vessels meander. In a mature vessel the basement membrane is mainly composed of laminin, collagen IV and perlecan [22-24], whereas in tissue repair the matrix also contains fibronectin, fibrin and vitronectin. Indeed, vascular stability heavily depends on the presence and composition of the ECM. The phenotype of mice deficient for individual basement membrane proteins reveals their necessity for vascular stability [25,26], as collagen IV- or perlecan knockout mice suffer from vascular instability and hemorrhage. However, this vascular instability may be attributed to defective vessel formation as well as to aberrant regulation of the endothelial barrier.

The involvement of the ECM in endothelial barrier regulation was stressed in a recent publication, which showed that changing stiffness of the ECM directly alters the barrier of the endothelium. Krishnan et al. [27] showed that endothelial cells grown on a soft ECM form substantially smaller gaps during stimulation with a barrier disruptive agent than endothelial cells grown on a stiff matrix. They further demonstrated that matrix stiffness is an important contributor to tension development in the cell. Intracellular tension, in turn, has a direct effect on the size and stability of cell-cell junctions, both under resting and stimulated conditions [28]. Increased ECM stiffness may result from metabolic conditions or vascular aging, and contribute to endothelial permeability as observed in atherosclerosis [29]. Details on the influence of extracellular matrix composition and geometry on endothelial barrier integrity are nicely surveyed in previous reviews [30,31].
These data support a clear, albeit chronic influence of the ECM on endothelial barrier integrity. The ECM is relatively static, and does not importantly change during the time interval of endothelial barrier disruption by vasoactive agents in acute inflammatory conditions (0-18h). However, the integrins that connect cell and matrix can be rapidly activated and subsequently activate regulatory signals into actin-associated changes within the cell. On the basis of this ability, integrins and the therewith associated signaling hub – the FAs – have been considered to contribute to endothelial barrier changes.

2.2. The role of integrins

Endothelial cells bind to the ECM via integrins. Integrins form a large family of glycoproteins that serve as cell adhesion receptors. These heterodimeric transmembrane proteins, composed of an alpha and a beta subunit, are the main adhesion receptors mediating adhesion of endothelial cells to the extracellular matrix. The large extracellular domain of integrins directly binds to the filamentous proteins of the ECM, while the small intracellular domain connects via other proteins to the actin skeleton, thus bridging the extracellular matrix with the endothelial cytoskeleton. Although the 18 alpha subunits and 8 beta subunits identified in vertebrates yield 24 different integrin heterodimers, only part of them is present in endothelial cells. The combination of alpha and beta subunits determines the affinity of integrins for a specific ECM molecule. In endothelial cells, the following alpha/beta heterodimers have been identified: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_9\beta_1$ (in particular lymphendothelial cells), $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [32-36]. Although integrins have been reported to be quite promiscuous with regard to binding of ECM molecules, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ preferentially bind collagen, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_9\beta_1$ bind laminin, $\alpha_5\beta_1$ fibronectin, whereas $\alpha_v\beta_3$ and $\alpha_v\beta_5$ bind vitronectin and many other proteins that contain the amino acid sequence Agr-Gly-Asp (RGD) [37,38].

Integrin-mediated binding of endothelial cells to the ECM importantly determines cell shape and morphology of the endothelial monolayer. Changes in integrin binding and expression parallel changes in cell shape as observed during formation and disruption of the endothelial barrier. Integrins are observed at the leading edge of spreading endothelial cells, whereas dissociation of integrin binding at the cell edge is required for cell retraction and gap formation. The reported contribution of integrins to endothelial barrier function is quite heterogeneous. Integrin subtype, endothelial cell type, the composition of the extracellular matrix and the presence of cytokines, growth factors or chemokines all contribute to this heterogeneity. Methods used to elucidate the contribution of integrins to endothelial barrier maintenance and endothelial barrier disruption include: RGD-containing peptides (which bind to integrins, thereby preventing integrin binding to the ECM), genetic mutation (functional inhibition) or ablation (either general or endothelial-specific) of integrins and integrin blocking or activating antibodies.

2.2.1. RGD peptides

Several endothelial integrins bind to the ECM at the specific amino acid sequence Agr-Gly-Asp (RGD). This RGD site is found in various integrin ligands, like fibronectin, vitronectin, laminin and collagen. RGD-peptides have been constructed that contain this sequence in a specific environment and therefore compete with integrin binding to (specific) ECM proteins [39]. Yet, it should be taken into account that RGD peptides may also affect intracellular signaling independent of the integrin/ECM binding [40]. Treating endothelial monolayers with RGD peptides resulted in a loss of barrier function, which was associated with reduced endothelial adhesion strength to the ECM [39]. This was also found in an *ex vivo* study of Wu et al., which demonstrated that inhibition of integrin binding to both fibronectin (with the fibronectin-specific RGD peptide, GRGDdSP) or vitronectin (with a vitronectin-specific peptide, GPenGRGDSPCA) increased the permeability of porcine venules. These effects were reversed by cotreatment with fibronectin or vitronectin, respectively [41]. From this perspective it is interesting that in humans the plasma fibronectin (but not soluble cellular fibronectin) is inversely related to mortality from acute lung injury or acute respiratory distress syndrome [42].

2.2.2. Integrin $\alpha_5\beta_1$

The integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ have been studied most extensively in the context of endothelial barrier regulation. Their effect on vascular leakage primarily reflect leakiness because of improper vessel development, but they were also found to act on established endothelial monolayers. Overall genetic ablation of the α_5 [43] or the β_1 [44] subunit or endothelial-specific ablation of the β_1 subunit [45] results in early embryonic lethality (at E10-11, E5.5 and E9.5, respectively) due

to failure of vascular development. The failed vascular development and early embryonic death of β_1 knockout mice most likely results from impaired $\alpha_5\beta_1$ heterodimer formation, as genetic ablation of the α_1 [46], α_2 [47], α_3 [48], α_6 [49] and α_9 [50] does not carry any defects in vascular development.

In resting, confluent endothelial monolayers $\alpha_5\beta_1$ is one of the most important integrins for maintenance of barrier function. Several *in vitro* studies have shown that treatment of confluent monolayers with an $\alpha_5\beta_1$-blocking antibody leads to formation of intercellular gaps and enhanced passage of macromolecules in a dose-dependent fashion [51,52]. The finding that coincubation with fibronectin (the natural ligand of $\alpha_5\beta_1$) reverses the barrier-disruptive effects of the $\alpha_5\beta_1$-blocking antibody [52] indicates that disruption of receptor-ligand interaction underlies the loss of barrier dysfunction.

Inhibition of $\alpha_5\beta_1$ may contribute to endothelial barrier disruption by inflammatory cytokines. Exposure of endothelial cells to TNF-α leads to enhanced endothelial permeability, associated with low presence of $\alpha_5\beta_1$ integrins at FAs [53]. TNF-α treated endothelial cells further show reduced adhesion to the ECM, due to increased internalization and recycling of $\alpha_5\beta_1$ integrins [54]. Treatment of endothelial cells with $\alpha_5\beta_1$-blocking antibody mimics TNF-α-induced cell detachment, without having additive effects when applied simultaneously, while treatment with an $\alpha_5\beta_1$-activating antibody or fibronectin attenuated the effects of TNF-α [55]. The relative contribution of disturbed cell-matrix interaction to TNF-α-induced endothelial barrier dysfunction remains to be elucidated, however, as effects of TNF-α on F-actin cytoskeleton and junctional integrity may be more prominent in disruption of the barrier [56]. Notwithstanding these studies together show that stable binding of $\alpha_5\beta_1$ to the extracellular matrix is required for endothelial barrier function, and that barrier-disruptive cytokines like TNF-α by targeting this cell-matrix interaction reduce endothelial monolayer integrity.

A new view on the involvement of $\alpha_5\beta_1$ in endothelial permeability was recently put forward. Besides binding to ECM molecules, endothelial barrier function may be affected by $\alpha_5\beta_1$ interaction with other proteins. cANGPTL4, a soluble C-terminal fibrinogen-like domain of angiopoietin-like 4, was found to bind the extracellular domain of $\alpha_5\beta_1$, leading to $\alpha_5\beta_1$ activation and internalization [57]. This $\alpha_5\beta_1$/cANGPTL4 interaction subsequently disturbed VE-cadherin/claudin-5 clustering and disrupted the endothelial barrier. An $\alpha_5\beta_1$-blocking antibody attenuated cANGPTL4-induced endothelial barrier dysfunction and vascular leakage, suggesting the involvement of $\alpha_5\beta_1$ in endothelial barrier disruption. The authors propose a mechanism in which cANGPTL4 binding to $\alpha_5\beta_1$ 1) induces Rac1/PAK driven endothelial barrier disruption, and 2) clears the way for cANGPTL4 binding to and disruption of VE-cadherin and claudin-5 complexes. The exact role of $\alpha_5\beta_1$ integrin with respect to cell-matrix interaction remains to be elucidated

here. Although cANGPTL4 does not contain an RGD-sequence (Source: PubMed Protein) and, according to the authors, cANGPTL4 does not impair $\alpha_5\beta_1$ binding to fibronectin [58], cANGPTL4 induces $\alpha_5\beta_1$ internalization, leaving less $\alpha_5\beta_1$ available for cell-matrix interaction. Another non-ECM protein reported to interact with $\alpha_5\beta_1$ is Tie2, the receptor for the angiopoietins Ang-1 and Ang-2. Binding of $\alpha_5\beta_1$ to Tie2 sensitizes Tie2 for Ang-1, a process that is further enhanced by $\alpha_5\beta_1$-binding to fibronectin [59]. Ang-1 binding to Tie2 increases endothelial barrier function *in vitro* [60], protects against edema formation in mouse models of acute lung injury (ALI), whereas plasma Ang-1 is inversely correlated with disease severity in sepsis and ALI [61].

In summary, $\alpha_5\beta_1$ and $\alpha_5\beta_1$/ECM-binding are required for maintenance of cell shape under physiologic conditions. Disruption of this $\alpha_5\beta_1$/ECM binding leads to swift dissociation of cell-matrix interaction, cell retraction and gap formation. This might involve enhanced recycling of $\alpha_5\beta_1$ in inflammatory responses. In contrast, boosting of $\alpha_5\beta_1$/ECM-binding (with additional fibronectin or integrin-activating antibodies) during cytokine stimulation attenuates endothelial barrier disruption, indicating that integrin-mediated cell-matrix interaction counteracts cell-retraction and gap formation. The interaction between $\alpha_5\beta_1$ and cANGPTL4 or Tie2 further demonstrates that binding of $\alpha_5\beta_1$ to cellular proteins can affect endothelial barrier function independent of cell-matrix interaction.

2.2.3. Integrin $\alpha_v\beta_3$

Unlike $\alpha_5\beta_1$, $\alpha_v\beta_3$ seems to be dispensible for vascular development and homeostasis. Although genetic ablation of the αv subunit is embryonically lethal at E9.5, due to severe cerebral hemorrhages [62], the lethal phenotype of α_v knockout mice most likely results from impaired formation of the $\alpha_v\beta_8$ heterodimer, which is required for end-feet association of perivascular cells with brain endothelial cells [38,63]. In line with this, mice with genetic ablation of the β3 subunit [64,65] or endothelium-specific ablation of the α_v subunit [66] show normal vascular development.

Studies on the role of $\alpha_v\beta_3$ integrin in endothelial barrier regulation have shown heterogeneous results, and have reported both barrier disruptive- and barrier-preserving effects of $\alpha_v\beta_3$ integrin. Blocking $\alpha_v\beta_3$ does not perturb the integrity of a resting endothelial monolayer. Mice lacking the β_3 show no edema or vascular leakage under non-stimulated conditions [67] and incubation of cultured endothelial cells with a $\alpha_v\beta_3$-blocking antibody does not impair endothelial barrier function [51,67]. In contrast, β_3 knockout mice or endothelial cells pretreated with an $\alpha_v\beta_3$-blocking antibody show enhanced vascular leakage and endothelial barrier disruption in response to stimulation with permeability-inducing agents [67,68]. Similarly, endothelial stimulation with TNF-α and IFN-γ reduced the $\alpha_v\beta_3$-dependent adhesion of endothelial cells to the extracellular

matrix [69]. These data suggest that $\alpha_v\beta_3$-mediated cell-matrix adhesion exerts a barrier stabilizing effect during endothelial stimulation. Li and Gamble have shown that stimulation of endothelial cells with thrombin directly activates signaling pathways that target and disrupt $\alpha_v\beta_3$-dependent cell-matrix interaction [68]. In addition, Thomas et al. [70] demonstrated that upon endothelial stimulation with Ang-2, $\alpha_v\beta_3$ forms a complex with Tie2 (an Ang-2 receptor) and is consequently internalized and degraded. As Ang-2 is generally known to induce [71,72] or prime for [73] endothelial barrier disruption, Ang-2 may use $\alpha_v\beta_3$ to induce endothelial monolayer destabilization and barrier dysfunction [74].

A number of studies has shown that $\alpha_v\beta_3$ contributes to endothelial barrier disruption. Measuring brain edema formation, Shimamura et al. [75] reported that $\alpha_v\beta_3$ inhibition with an $\alpha_v\beta_3$-specific RGD sequence (cRGDfv) attenuated brain edema following stroke. In line with this finding, the $\alpha_v\beta_3$ antagonist BS-1417 reduced vascular leakage resulting from choroidal neovascularization [76], while αvβ3 blockade (*in vitro*) or genetic αvβ3 ablation (*in vivo*) attenuated endothelial barrier disruption induced by fibrinogen-γ C-terminal fragments [77]. The interaction of $\alpha_v\beta_3$ with the VEGFR2, as shown by Soldi et al. [78] further supports the involvement of $\alpha_v\beta_3$ in endothelial barrier disruption. $\alpha_v\beta_3$ binding to its ligand vitronectin is required for phosphorylation and activation of the VEGFR2 upon binding with its ligand VEGF-A165, suggesting that $\alpha_v\beta_3$/VEGFR2 complex formation is a prerequisite for VEGF-induced vascular leakage.

Multiple explanations may underlie the paradoxical role of $\alpha_v\beta_3$ in endothelial barrier regulation. As large differences exist between the regulation of blood-brain barrier function and barrier function in the rest of the endothelium, $\alpha_v\beta_3$ may act differently in brain endothelial cells, in particular in combination with astrocyte end-feet. In addition, extracellular modulation of $\alpha_v\beta_3$ by for example ECM proteins or peptides (outside-in signaling) differently affects integrin function than intracellular signals, due to agonist-induced activation (inside-out signaling).

2.2.4. Integrin $\alpha_v\beta_5$

Considerably less is known about the involvement of other integrins in endothelial barrier regulation. In a series of publications Pittet et al. pointed towards a role of $\alpha_v\beta_5$ in mediating the pathogenesis of acute lung injury and pulmonary edema. Blockage of $\alpha_v\beta_5$ and $\alpha_v\beta_6$ attenuated IL-1β-induced disruption of lung endothelial cell monolayers, and reduced pulmonary edema formation during acute lung injury resulting from IL-1β overexpression in murine lung [79]. Similarly, $\alpha_v\beta_5$ blocking antibodies attenuated endothelial barrier disruption by VEGF, thrombin and TNF-α, whereas genetic ablation of the β_5 subunit attenuated lung vascular permeability resulting from ventilator-induced lung injury [80]. As $\alpha_v\beta_5$ was also shown to be involved in pulmonary edema formation related to *Pseudomonas aeruginosa* pneumonia [81], these

studies indicate $\alpha_v\beta_5$ as an important and general mediator of endothelial barrier disruption and pulmonary edema formation during acute lung injury.

2.2.5. Other integrins

Although a direct association of $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_6\beta_1$ integrins with endothelial barrier disruption or edema formation is lacking, indirect evidence may suggest their involvement in endothelial barrier regulation. Cailleteau et al. [82] have shown that $\alpha_2\beta_1$ activity prevents Rac1 anchorage to the cell membrane, thereby hampering endothelial cell quiescence, a condition required for proper barrier function. Integrin $\alpha_3\beta_1$ localizes to endothelial cell-cell junctions and regulates endothelial cell migration and cell-matrix adhesion – $\alpha_3\beta_1$ blocking antibodies inhibit endothelial cell migration and increase cell-matrix interaction [83]. $\alpha_4\beta_1$ blocking antibodies attenuate diabetes-induced upregulation of VEGF, TNF-α and NFκB, leukocyte adhesion and vascular leakage [84]. However, even though $\alpha_4\beta_1$ is present in endothelial cells, $\alpha_4\beta_1$ is generally considered as a leukocyte-specific integrin [24]. For this reason, the protecting effects of $\alpha_4\beta_1$ can also be attributed to $\alpha_4\beta_1$ inhibition on leukocytes. Last, $\alpha_6\beta_1$-deficient endothelial cells show increased VEGFR2 expression and VEGF-dependent signaling, whereas genetic ablation of $\alpha_6\beta_1$ in mice leads to enhanced angiogenesis [85]. As stated before, no direct relation to endothelial barrier disruption is known for these integrins. Together, the presence or activity of these integrins seems to directly modulate pathways that affect endothelial barrier integrity. The precise involvement in endothelial barrier regulation remains to be elucidated, however.

2.2.6. Conclusion integrins

These findings stress the relevance of integrins in endothelial barrier regulation, although the role of integrins role is quite heterogeneous. Interaction of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ with the ECM clearly protect the endothelial barrier, whereas $\alpha_v\beta_5$ contributes to endothelial barrier disruption. In addition, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins may interact with other proteins than ECM proteins. Integrin binding results in intracellular signaling and structural changes, which in turn affect endothelial barrier function. The involvement of the remaining endothelial integrins in barrier regulation is less clear. Although knock-out models, either whole-body or endothelial-specific, have yielded considerable insight in the role of integrins in vasculatur development and angiogenesis, the effect on endothelial barrier regulation is difficult to extract from these studies.

2.3. Focal adhesions as signaling hub of matrix-integrin interaction

Inside the cell integrins bind to the actin cytoskeleton via the FA, a large multiple-protein complex containing scaffold proteins, kinases, phosphatases and a large number of GTPases [86,87]. The core of this integrin-cytoskeleton connection is formed by talin and vinculin (Figure 2). The binding of integrin via talin and vinculin to actin is regulated by allosteric changes in talin and vinculin conformation. The head domain of talin binds to the cell membrane and to the cytoplasmic domain of the integrin in a $PI(4,5)P_2$-dependent manner. The talin tail domain binds to the actin cytoskeleton inducing tension and unfolding of talin. This stretched allosteric conformation of talin uncovers binding sites for vinculin, which crosslinks talin and actin and enhances the integrin/talin/actin connection. In addition, vinculin activation (separation of its head and tail regions) enhances FA growth by clustering of activated integrins and slowing down FA turnover [88]. The FA protein complex is further enriched with structural proteins like tensin, α-actinin and kindlin, and adaptor proteins like paxillin (Figure 2). In the FA paxillin facilitates cell signaling from integrin/ECM to the cytosol and vice versa [89]. Proteins involved in these signaling pathways are the tyrosine kinases Src and Focal Adhesion Kinase (FAK). FAK binds to paxillin and talin, and is activated by autophosphorylation upon ECM binding or by phosphorylation by Src. Additional signaling is mediated by the RhoGTPases Rac1, Cdc42 and RhoA. Apart from these classical and well-characterized members, the FA protein complex counts many other proteins, altogether forming a complex from over 50 proteins [90].

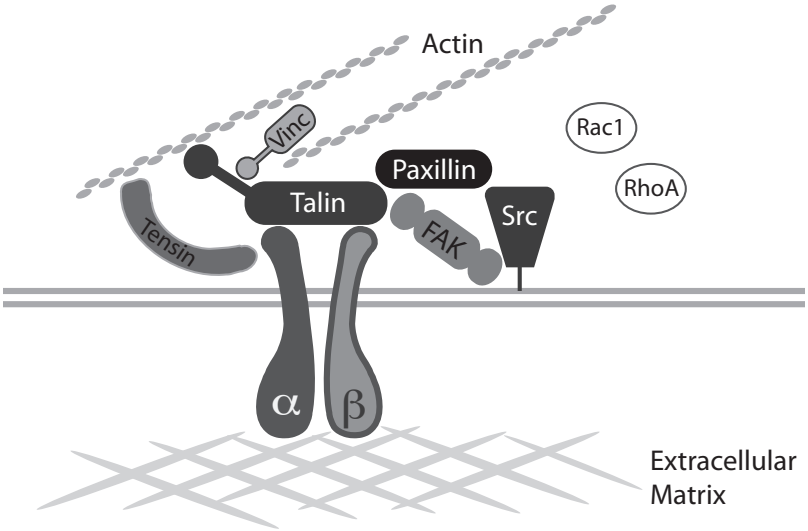

Figure 2 Schematic representation of the focal adhesion. Proteins most important for cell-matrix interaction and its regulation are presented here. α and β represent integrin subunits. The RhoGTPases RhoA and Rac1 importantly contribute to focal adhesion dynamics, but do not specifically bind to one protein.

2.3.1. Contribution of individual FA proteins to endothelial barrier regulation.

The involvement of the FA in endothelial barrier regulation can be considered in two ways: a) by looking at the role of the individual FA proteins in endothelial barrier regulation, and b) by looking at the role of the complex as a whole. Although most FA proteins have been characterized in mouse knockout models (excellently reviewed in [90]), little is known on their vascular effects. Genetic ablation of proteins like talin, vinculin and paxillin induces embryonic lethality between E8.5 and E10, strongly suggestive for impaired vascular development, but as far as we know this has not been confirmed in endothelial-specific knockout models. In addition, little is known about the effect of genetic depletion of structural proteins like vinculin and talin in cultured endothelial monolayers. Kindlins activate integrins, and as such may contribute to endothelial barrier regulation [91]. Kindlin-2$^{+/-}$ mice show increased basal endothelial permeability, but show no additive permeability response to VEGF [92].

The opposite is true for the regulatory proteins Src, FAK and the RhoGTPases. In particular the role of FAK in endothelial barrier regulation has been extensively addressed in the last decade. The regulation of FAK and its functional consequences during endothelial barrier maintenance and disruption are extremely complex. The complexity of spatial and temporal regulation may count for the fact that studies indicate both barrier-disrupting [93] and barrier-protective effects of FAK [94]. The role of FAK in endothelial barrier regulation is addressed in a series of recent reviews on this topic [95-97]. Likewise, the role of Src and the RhoGTPases have been extensively studied in the role of endothelial barrier regulation (for review see [2]). Yet, these proteins – although frequently present in FAs – are found throughout the whole cell. The effect of modulating their action is not limited to changes in FA function, but also affects actomyosin contraction and AJ dynamics.

Studying the role of FAs in endothelial barrier regulation by targeting individual FA proteins is limited by decreased cell viability upon inhibition of essential FA proteins, redundancy of proteins in the FA (loss of function of a specific FA protein is compensated by other proteins), and altered functionality in other parts of the cell in case the presence of a protein is not limited to FAs [21]. Alternatively, the involvement of FAs in endothelial barrier regulation can be studied by following these complexes as a whole (mainly immunofluorescence or live cell imaging) during changes in endothelial barrier function.

2.3.2. Involvement of FAs in endothelial barrier regulation.

A hallmark study in the regulation of FAs has been the 1992 paper of Ridley and Hall [98]. They show that stimulation of fibroblasts with the growth factor PDGF leads to fast formation of actin stress fibers and FAs. Both events could be abrogated by transfection of fibroblasts with

a dominant negative RhoA mutant. Later studies have demonstrated that similar processes are displayed during endothelial barrier disruption [99,100]. Stimulation of confluent endothelial monolayers with permeability-inducing factors like VEGF, thrombin and TNF-α induces rapid formation of stress fibers and FAs, associated with cell contraction. The close spatio-temporal relation between stress fiber formation, FA assembly, actomyosin contractility, cell retraction and endothelial barrier disruption suggested that these processes form a chain of events, in which each event plays a mediating role. This was supported by the finding that during endothelial barrier disruption stress fibers are capped at both ends with thick and dense FAs [98,101], giving rise to the general idea that FAs form anchoring points for stress fibers. Stress fibers, thus firmly attached to FAs, can exert pulling force on the cell periphery (in particular the AJ), leading to AJ disruption, cell retraction and gap formation [100,102].

This idea, however, seems difficult to match with the earlier discussed work on integrins, which showed that integrins, as the transmembrane part of the FA, are required for maintenance of endothelial barrier function ($\alpha_5\beta_1$), and that disturbed integrin binding can enhance endothelial barrier disruption ($\alpha_v\beta_3$). Further evidence against the idea that FAs mediate endothelial barrier disruption came from the work of Birukov and Garica on barrier-enhancing phospholipids [30. Romer et al. 2006]. They demonstrated that endothelial barrier enhancement with S1P and oxidized phosphoplipids is associated with an increase in the amount of FAs, in particular at the periphery of the cell [101,103]. These peripheral FAs stay in close contact with and seem to support the cortical actin band, a dense ring of actin fibers located next to the cell membrane and characteristic of a resting endothelial monolayer [104,105]. The phospholipid-induced relocalization of FAs was shown to be Rac1 dependent [103,106], which agrees with other studies that show involvement of Rac1 in the new formation of FAs at the cell periphery during cell spreading [107,108]. These studies suggest that the presence of FAs contributes to endothelial barrier function and prevents further disruption of the barrier during endothelial stimulation. In the majority of these studies FA dynamics were studied by immunofluoresence microscopy, and correlated in time to effects on endothelial barrier function, predominantly yielding associative conclusions and leaving open causal questions. These merely associative answers, together with the limitations on studying FA dynamics in endothelial barrier regulation discussed above, resulted in the comment that "*…the specific contribution of focal adhesions to the regulatory mechanism of microvascular barrier function remains a mystery…*" [21].

However, the difference in FA location during endothelial junction disassembly (FAs located predominantly at ends of actin stress fibers) and endothelial barrier enhancement (FAs located predominantly at the cell periphery) suggests an important role of FA spatial distribution in endothelial barrier regulation.

2.3.3. Subcellular localization of FAs associated with endothelial barrier function

The relation between the spatial distribution of FAs and endothelial barrier function can be predicted by the tensegrity model as described by Ingber [109]. According to this model, cells can only maintain their shape when the internal tension is balanced by distension through compression-resistant elements or through extracellular adhesions. When translating this principle to endothelial cells in a monolayer, one can say that the cell shape as required for monolayer integrity (spread cell morphology) is maintained by a low internal tensile force (low actomyosin contraction) counterbalanced by amongst others FAs. Presence of FAs only at the center of the cell is not sufficient to maintain the spread morphology, but induce cell retraction and rounding up. However, the presence of FAs at the periphery is sufficient to maintain the spread shape of the cell required for monolayer integrity [110,111]. In addition, peripheral redistribution of FAs not only maintains cell shape, but also enhances cell adhesion strength to the ECM, as demonstrated in an elegant study of Elineni and Gallant [112]. Seeding fibroblasts on fibronectin islands with a solid circular versus an annular shape, they demonstrated that cells grown on an annular fibronectin island have a higher adhesion strength than cells grown on solid circular islands, even though the adhesive area was constant. Although these studies need confirmation in endothelial cells, they indicate that peripheral redistribution of FAs during endothelial barrier restoration or fortification contributes to improved endothelial barrier function. Otherwise, preservation of peripheral FAs during endothelial stimulation may attenuate gap formation and barrier dysfunction.

Two issues should be taken into account when considering spatial distribution of FAs, a theoretical and a methodological issue. The theoretical issue is the bidirectional signaling in FAs – FAs convert ECM-dependent integrin conformation to intracellular signaling (outside-in signaling), but also converts intracellular signals to changes in integrin conformation (inside-out signaling). Outside-in signaling is required for sensing of the extracellular environment and the consequent intracellular response [110], while inside-out signaling mediates growth factor-induced changes in cell adhesion. Both pathways make use of the same FA proteins, but may differently affect endothelial barrier function. The methodological issue is the identification of FAs. In the past several proteins have been used to identify FAs, as these proteins were supposed to be FA-specific (e.g. FAK and vinculin), and to study cell-matrix interaction in particular with immunofluoresence staining. This approach carries two problems: a) it is not clear how the spatial distribution of these single proteins relates to true cell-matrix receptors (integrins) and to true cell-matrix adhesive force, and b) ongoing research has doubted the FA-specificity of these proteins. Vinculin, for example, was recently shown to be also present in AJs [16,113]. These problems warrant care in the interpretation of experiments in which proteins like FAK and vinculin are used to identify FAs or cell-matrix interaction.

2.4. Conclusion

From the foregoing two mechanisms come forward explaining the role of integrins and FAs in endothelial barrier function. First, reinforcing cell matrix adhesion, in particular at the cell periphery, compensates the loss of tethering force due to dissociation of cell-cell junction. Secondly, integrin-activation causes intracellular signaling (FAK, Src, Rho GTPases and other) and altered F-actin dynamics, which may directly or indirectly affect the intracellular tension dynamics and cell junction proteins. Various proteins that are classically associated with FAs cluster in the region of the AJ, in particular immediately after destabilization of these junctions by vasoactive and inflammatory stimuli.

3 – AJ proteins reinforce cell-matrix interaction during endothelial barrier disruption

3.1. The Adherens Junction Structure

Endothelial AJs resemble FAs in that they form a connection between the extracellular environment (in this case neighbouring cells) and the cytoskeleton (Figure 3). As indicated above, the core of the AJ is the transmembrane protein VE-cadherin, which binds in a homophilic, calcium-dependent fashion to VE-cadherin of the neighbouring cell. Inside the cell VE-cadherin binds to three members of the catenin family. α-Catenin and β-catenin directly or indirectly bind VE-cadherin and both mediate the attachment of actin to the AJ. Attachment to the actin cytoskeleton is required for AJ stabilization as absence or dysfunction of these catenins hampers vascular development. The third is p120-catenin which is predominantly involved in binding phosphatases to the AJ. Phosphatases keep VE-cadherin and β-catenin in a dephosphorylated state, and since tyrosine phosphorylation of VE-cadherin and beta-catenin is an initial event in AJ dissociation [9], p120-catenin further stabilizes the AJ.

A number of proteins present in mature or destabilized AJs is also found in FAs. Among these proteins are vinculin, p190RhoGAP, VASP and FAK. Other proteins are neither AJ- nor FA-specific, but show a remarkable translocation from the one site to the other during endothelial barrier disruption or recovery. Examples of these proteins are the regulatory RhoGTPases Rac1 and RhoA. The next paragraphs will discuss the role of vinculin, p190RhoGAP and other proteins in AJ and FA regulation during endothelial barrier disruption. During endothelial barrier disruption, these proteins relocate from the dissociating AJ to the FA. Stimulated endothelial cells show a large increase in the number of FAs – FA formation and reinforcement of cell-matrix interaction may therefore compensate the loss of tethering forces due to AJ dissociation.

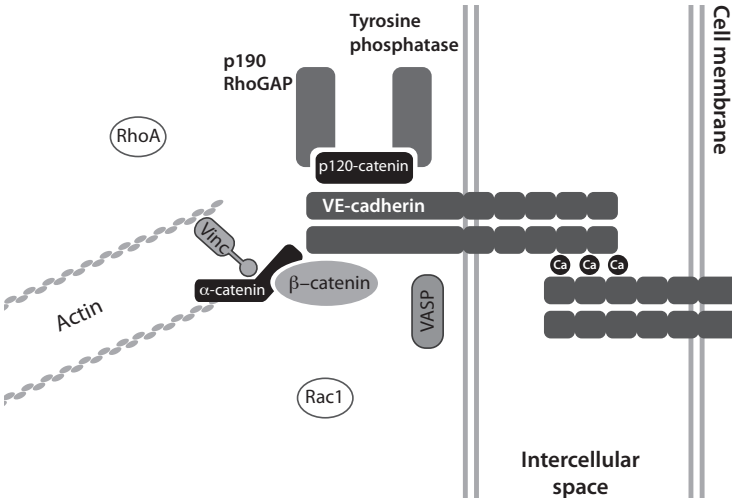

Figure 3 Schematic presentation of an adherens junction in the endothelium. Calcium-dependent homophilic VE-cadherin binding between neighbouring cell forms the core of this complex. VE-cadherin is a transmembrane protein that binds inside the cell to the actin cytoskeleton via a number of linker proteins. In addition, VE-cadherin binds to scaffolding proteins like p120-catenin that link regulatory proteins like phosphatases and p190RhoGAP.

3.2. Vinculin

Vinculin, a classical FA protein, was already demonstrated to be present in cell-cell junctions decades ago [51,114], although its function in AJs remained poorly understood. Studies in both epithelial and endothelial monolayers have made fast progress in this area and indicated a clear role for vinculin in stabilization of AJs [113,115]. In a resting monolayer, AJs are subject to low actomyosin tension required for AJ stability. This tension induces a slight allosteric change in α-catenin configuration, which uncovers binding sites for vinculin [116,117], similar to the tension-induced allosteric changes in talin which facilitate vinculin incorporation in the FA. In addition to its AJ-stabilizing effect under non-stimulated conditions, the presence of vinculin in AJs was recently shown to attenuate endothelial barrier disruption by thrombin, VEGF and TNF-α. Vinculin makes AJs more resistant to tensile forces exerted on the junction during actomyosin contraction. Mutation of the vinculin binding site on α-catenin (which prevents vinculin recruitment to the AJ) enhances gap formation upon agonist-induced actomyosin contraction [16].

In a resting monolayer and during the early phase of barrier disruption vinculin localizes predominantly at the cell perimeter (suggestive for its presence in the AJ). The vinculin present in the cytosol has an inactive configuration. During prolonged stimulation with barrier disruptive agents vinculin disappears from the AJs and accumulates in FAs. Sakurai et al. [118] showed that acute disruption of the VE-cadherin bond with the calcium-chelator EGTA leads to an immediate

loss of vinculin from the cell perimeter and consequent vinculin accumulation in FAs. Reversing this condition by calcium resuppletion resulted in fast (within 20 min) disappearance of vinculin from FAs and restoration of vinculin at the membrane. As changes in expression are precluded by the short interval of stimulation, this study is strongly suggestive for a common pool of vinculin serving both FAs and AJs. Even more, the relocalization of vinculin from the dissociating AJ to the FA readily explains the reinforcement of cell-matrix interaction upon loss of cell-cell adhesion.

3.3. p190RhoGAP

Like vinculin, p190RhoGAP is a protein present in both AJs and FAs. p190RhoGAP is a GTPase Activating Protein (GAP) that inactivates RhoA by converting it from a GTP- to a GDP-bound state. p190RhoGAP activity is regulated by tyrosine phosphorylation and nitration. In endothelial cells, p190RhoGAP is observed in AJs where it locally inactivates RhoA, thereby attenuating RhoA-dependent actomyosin contraction in the junctions. The presence of p190RhoGAP in the AJ is required for maintenance of AJ integrity [119], and redistribution of p190RhoGAP to the membrane mediates the effects of barrier-enhancing agents like oxidized phospholipids [120]. In contrast, during endothelial barrier disruption with thrombin, p190RhoGAP is nitrated and inactivated, leading to increased RhoA activity in the AJ, and disruption of the AJ [121].

In addition to the AJ, p190RhoGAP is active in the cytosol, in particular at sites of cell-matrix adhesion. p190RhoGAP colocalizes with paxillin at peripheral FAs, and knockdown of p190RhoGAP prevents the formation of peripheral FAs [122]. As RhoA inhibits cell spreading and initial FA formation [123], local inhibition of RhoA by p190RhoGAP is required for formation of new FAs and cell spreading. Impaired 190RhoGAP localization to FAs reduces FA turnover, leading to formation of large FAs, probably due to enhanced and prolonged local RhoA activity [124]. The spread phenotype, due to peripheral localization of FAs and stable AJs, is characteristic of Rac1 activity. The opposite phenotype – a round-up morphology with disrupted AJs and contractile stress fibers connected to large FAs characterizes cells with high RhoA activity. In between p190RhoGAP acts a central regulator of the antagonism between Rac1 and RhoA as already described for epithelial cells [125]. Membrane-bound p190RhoGAP may thus enhance AJ stability in the resting endothelium, whereas cytosolic, FA-bound p190RhoGAP enhances FA turnover and cell spreading in recovering endothelium. Although these observations strongly suggest a mechanistical link between AJ and FA regulation, evidence for such a link is less established than for vinculin. Live cell tracking of p190RhoGAP during endothelial barrier disruption and recovery is required to confirm this circumstantial evidence. As Rac1 [120,125] and Focal Adhesion Kinase (FAK) [119,122,126,127] both regulate p190RhoGAP activity, and both are present in FAs and AJs, dynamics of these proteins may further clarify the cell-matrix reinforcement upon AJ disruption [128].

3.4. VASP

A third protein involved in the regulation of both the AJ and the FA is VASP. In confluent cells VASP localizes at stabilizing junctions [4], as already discussed in section 1.2., and at stable AJs contain VASP. When endothelial cells grow confluent, more and more VASP is recruited to AJs. Here, VASP contributes to AJ stability and endothelial barrier function by enhancing the formation of the cortical actin band [129]. VASP is required for stability of the endothelial barrier, as demonstrated by reduced endothelial barrier function of VASP-deficient mice [130] and VASP-depleted endothelial cells [131]. In sparsely seeded cells [129,132] or cells stimulated with vasoactive agents [133], however, VASP is found in FAs. The latter study demonstrated that the thrombin-induced translocation of VASP from AJs to FAs is mediated by zyxin. Depletion of zyxin enhanced the thrombin-induced endothelial barrier disruption and retarded recovery after thrombin stimulation [133]. These studies indicate that relocalization of VASP from the AJ to the FA during thrombin stimulation protects against endothelial barrier dysfunction. This may be explained by two mechanisms. First VASP may attenuate cell retraction and gap formation by actin polymerization and cytoskeletal reinforcement: at the level of the AJ (by forming the cortical actin band and the consequent AJ stabilization) in resting, confluent cells, or at the level of the FA (by stabilizing the contact of FAs with the cytoskeleton) in sparse of stimulated cells. Second, as shown by Schlegel and Waschke [134], VASP is required for integrin-mediated adhesion. This study suggests that upon AJ dissociation VASP may reinforce integrin-mediated adhesion.

3.5. Conclusion

Altogether, these studies demonstrate a close relation between loss of cell-cell contact and reinforcement of cell-matrix interaction. FAs and AJs share proteins that have a similar function in both protein complexes (e.g. in both the AJ and the FA vinculin links the transmembrane receptor to the actin cytoskeleton in a manner which is dependent on tension-induced allosterical changes in another linker protein). Assuming that a common pool serves both complexes, loss of protein from the one complex (in this case AJ) may lead to accumulation of that protein in the other complex (in this case FA). The increased tethering force in the FA (due to FA reinforcement) thus compensates the loss of tethering force in the AJ (Figure 4).

As we have seen before, cell shape and endothelial barrier function depend on tethering forces in cell-cell contacts, actomyosin contractile force and adhesive force to the ECM. Under basal conditions (in a resting endothelial monolayer) the sum of these forces is zero, resulting in a spread endothelial cell and an intact endothelial barrier. Upon stimulation with permeability factors [1] there is an acute increase in actomyosin contraction and dissociation of the cell-cell junctions.

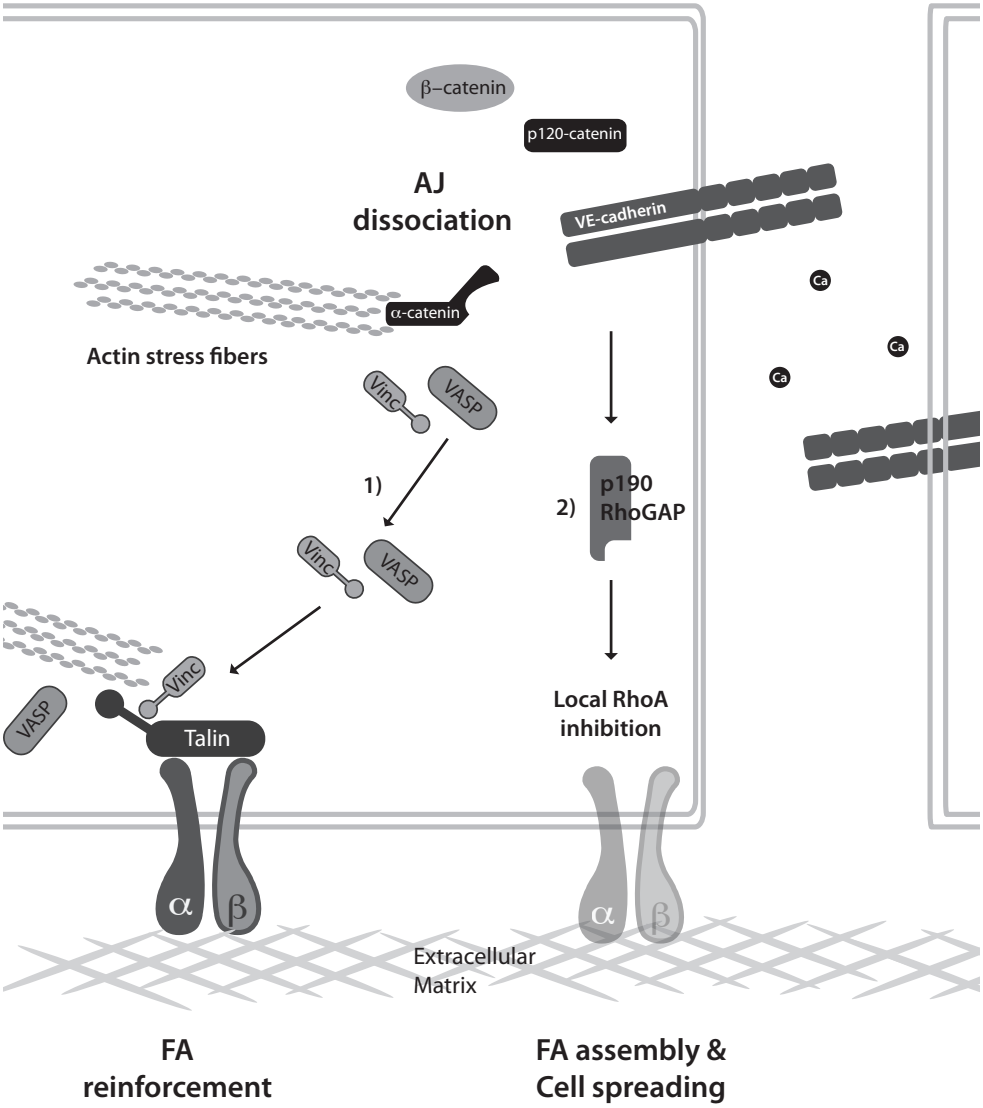

Figure 4 Possible mechanisms for focal adhesion reinforcement upon adherens junction dissociation. As described in the text, two mechanisms may mediate reinforcement of binding of the cell to the extracellular matrix. Upon adherens junction dissociation: 1) Vinculin and VASP are released in the cytosol and relocates to focal adhesion, where it stabilizes focal adhesions and increases binding strength to the extracellular matrix, and 2) p190RhoGAP is released in the cytosol and may contribute to formation of novel focal adhesions and cell spreading by local RhoA inhibition.

The resulting dysfunction of the endothelial barrier is determined by the size of the intercellular gaps. The size of the intercellular gaps, in turn, is determined by the extent of cell retraction. In the absence of functional cell-cell junctions the extent of cell retraction mainly depends on the adhesion of cell edges to the ECM. The influence of cell-matrix interaction (reflected by the number of FAs) on cell retraction, and thus on endothelial barrier disruption, is illustrated in Figure 5. A low number of FAs at the cell periphery facilitates swift cell retraction and formation of large intercellular gaps (Figure 5A), whereas a high number of peripheral FAs prevents cell retraction, and reduces the size of the intercellular gaps (Figure 5B). Reinforcement of cell-matrix interaction by either preservation of existing FAs or formation of new FAs may therefore serve as a 'hand brake' that prevents rigorous retraction of the cell. An increase in tethering force in the FAs thus forms a compensation for the loss of tethering force at cell-cell junctions during endothelial barrier disruption.

A clear illustration of such a protective compensation between tethering forces in cell-cell contacts and cell-matrix interaction was recently shown for epithelial cells. Lehembre et al. [135] showed that loss of E-cadherin resulted in upregulation of NCAM. NCAM and E-cadherin were reciprocally expressed, and the increased expression of NCAM induced assembly of β1-integrin-dependent FAs [135,136].

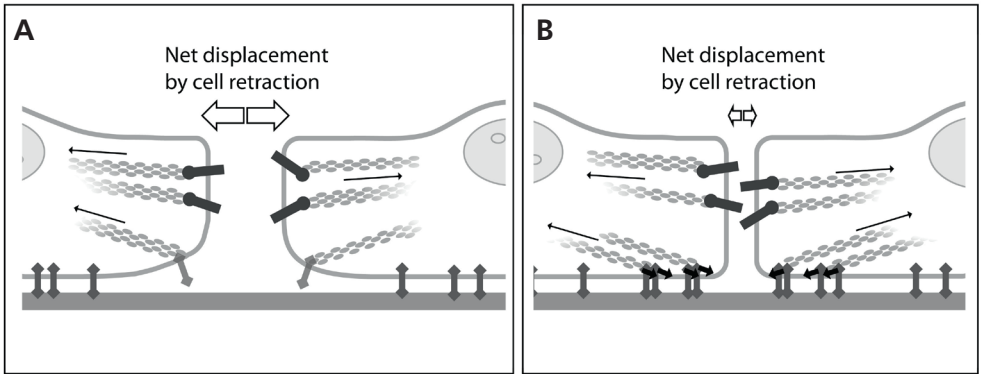

Figure 5 Overview of forces observed in endothelial cells within an endothelial monolayer stimulated with barrier-disruptive agents. Stimulation of endothelial cells with barrier disruptive agents leads to dissociation of adherens junctions and increased actomyosin contraction. Consequently intercellular gaps are formed, the size of which depends on tethering forces in cell-matrix adhesions. A) Low tethering forces in cell-matrix adhesions facilitates cell retraction, resulting in larger net cell displacement and intercellular gaps. B) High tethering forces in cell-matrix adhesions hampers swift cell retraction and limits the net cell displacement.

4 – CONCLUSIONS AND FUTURE PERSPECTIVES

4.1. Summary of the proposed model

In summary this chapter illustrates the significance of proteins involved in cell-matrix interaction for endothelial barrier regulation. Binding of cells to the ECM is required for maintenance of the spread cell shape that characterizes a functional monolayer. As a tethering force counteracting actomyosin contraction cell-matrix interaction protects against endothelial barrier disruption. During endothelial barrier disruption cells may reinforce their adhesion to the ECM as a defensive mechanism to protect against rigorous cell retraction. Attenuating cell retraction thus diminishes gap formation and prevents cell rounding up (and thus apoptosis). This reinforcement of cell-matrix interaction is closely related in time to AJ disruption. Reviewing dynamics of vinculin, p190RhoGAP and VASP, we propose that AJ disruption and FA reinforcement are mechanically linked. The observations that 1) enhanced cell-matrix interaction attenuates endothelial barrier disruption, and that 2) AJ disruption is mechanically linked to FA reinforcement, support the idea that reinforcement of cell-matrix interaction is a defensive mechanism by which endothelial cells protect the monolayer against barrier dysfunction (Figure 5).

4.2. Remaining questions

Yet, several important issues remain unresolved, first of which is integrin signaling during endothelial barrier regulation. Although a lot is known about the functional relevance of integrins in endothelial barrier regulation (as elucidated by knockout models, RGD peptides and integrin function modulating antibodies), little is known about the regulation of integrin function during agonist-induced endothelial barrier disruption. To the best of our knowledge there are very few studies that address the effect of permeability on the spatiotemporal distribution of integrins, the changes in integrin affinity and avidity for ECM ligands, or on integrin internalization and recycling. Integrin activation may play a central role in these processes. As known from circulating blood cells like platelets or leukocytes, inflammatory cytokines can activate or deactivate integrins by so-called inside-out signaling. Cytokine receptor activation induces intracellular signaling, leading to changes in integrin conformation, which in turn changes affinity and avidity of integrins for their ECM ligands, and thus affect the binding of endothelial cells to the ECM. Evaluation of integrin activity (with antibodies specific for epitopes uncovered in activated integrins) upon endothelial stimulation with permeability factors will contribute to the understanding of integrin regulation during vascular leakage and edema formation.

A second point, closely related to this matter, is the composition of FAs during endothelial barrier maintenance and disruption. Most studies have considered FAs as rather uniform in composition,

but functional analyses of cell-matrix interaction and barrier function raise doubts about this approach. This FA heterogeneity is particularly illustrated by two observations discussed in this chapter: 1) integrins play a heterogenous role in endothelial barrier regulation – $\alpha_5\beta_1$ and $\alpha_v\beta_3$ usually protect against endothelial barrier disruption, whereas $\alpha_v\beta_5$ evidently contributes to endothelial barrier disruption, and 2) during endothelial barrier disruption some FAs bind to stress fibers, whereas other FA-like complexes are observed independent of stress fibers, at the periphery of the cell. Together with spatial distribution, the idea of heterogeneous FA composition explains and combines both observations: central, stress fiber-bound FAs containing 'deleterious' integrins like $\alpha_v\beta_5$ contribute to endothelial barrier disruption, whereas peripheral FAs containing 'beneficial' integrins like $\alpha_5\beta_1$ maintain basal barrier function, attenuate barrier disruption and mediate barrier restoration.

4.3. Therapeutic implications

Given these data, it is striking that a drug like volociximab (an FDA-approved $\alpha_5\beta_1$-blocking antibody designed to inhibit integrin-mediated tumor angiogenesis [137]) gained fast access to clinical trials. The barrier-disrupting properties of $\alpha_5\beta_1$-blockage observed in *in vitro* studies may give rise to edema in clinical setting, in particular patients prone to edema because of increased systemic inflammation (like sepsis and acute lung injury). Indeed, clinical trials on volociximab reported (peripheral) edema as side-effect in 10-35% of treated patients [138, http://www.evaluatepharma.com/Universal/View.aspx?type=Story&id=86561].

From a clinical point of view, the data reviewed in this chapter indicate that proteins involved in cell-matrix interaction may form a target to attenuate endothelial barrier disruption and vascular leakage. In the search for specific treatment, improvement of integrin binding to the ECM may be a suitable option, as interfering with e.g. vinculin or RhoGTPase dynamics will interfere with cell-matrix independent processes. Enhancing cell-matrix binding may seal the endothelial barrier to prevent it against inflammatory charges, or reinforce the defensive response to treat barrier disruption.

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