The Role of VEGF in Controlling Vascular Permeability

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Abstract

Blood vessels in different organs have vastly different morphologies and functions. One important aspect of vessel heterogeneity is its exchange with the surrounding tissue. While vessels in the CNS are highly restricted in their

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exchange, vessels in peripheral organs may be quite permeable and allow solvent and small molecules to pass across the vessel wall. A more extensive permeability, or leakage, can be induced in an acute, transient manner by specific factors, with the purpose to deliver blood constituents to the interstitial space. The interstitial fluid is drained by the lymphatic vasculature and eventually delivered back to the blood circulation via the subclavian veins. Larger volumes of accumulated interstitial fluid, edema, are a sign of extensive leakage and/or poor uptake of fluid by the lymphatics. Through the continuous blood and lymphatic circulation, the maintenance of tissue homeostasis is ensured through the delivery of oxygen and nutrients to the tissues. In pathologies, the vasculature is often affected by, and engaged in, the disease process. This may result in excessive formation of new, unstable, and leaky vessels with poor blood flow and tissue swelling potentially exacerbated by poorly functioning lymphatics. Elevated interstitial pressure, hypoxia, and a chaotic tissue microenvironment promote the disease. This review is focused on the role of vascular endothelial growth factors (VEGFs) and their receptors in the control of vessel integrity.

Keywords
VEGF · Permeability · Edema · Flow · Pore · Junction

Introduction

Endothelial cells, key constituents of blood and lymphatic vessels, rest with their basolateral side on a specialized basement membrane, while their apical side faces the blood and the lymph, respectively. The main function of the blood vasculature is to serve as a conduit for the blood to ensure efficient oxygenation and nutrition of tissues. The lymphatic vasculature is pivotal for a range of homeostatic functions such as maintenance of a physiological interstitial pressure, lipid transport, and immune surveillance (Lanitis et al. 2015).

The blood vasculature is stable and its endothelial cells turn over very slowly in the healthy individual (Lee et al. 2007). During particular physiological responses such as embryo development, ovulation, and regrowth of the endometrium, or in conjunction with injury or disease, there is a need for new vessels to form, to nurture the growing or regenerating tissue. In fact, the growth of all new tissues, whether healthy or not, is accompanied by blood vessel formation, neoangiogenesis. A main underlying mechanism in neoangiogenesis is the relative hypoxia in the growing tissue (Liao and Johnson 2007), which drives expression of a wide range of growth factors including vascular endothelial growth factor (VEGFA), described below. VEGFA is essential in stimulating formation of new vessels and in survival of existing ones (Simons et al. 2016). During embryogenesis, vessels form de novo in a process denoted vasculogenesis, while angiogenesis implies vessel formation from the preexisting vasculature. The newly formed vasculature undergoes remodeling, also denoted "pruning," to form a hierarchical order consisting of arteries, capillaries, and veins. Pruning may involve apoptosis, cell death, of endothelial cells in vessels that lack flow; alternatively, it depends on the local motility of endothelial cells to reshape the vessels to new dimensions and densities to meet the needs of the tissue (Korn and Augustin 2015).

Blood vascular endothelial cells in different blood vessels and in different organs have distinct functions, and display in part, unique gene expression patterns (Augustin and Koh 2017). Thus, certain molecules such as neuropilins and members of the Eph family of receptor tyrosine kinases are preferentially expressed in arteries and not in veins, while expression of other molecules is restricted to veins. Other distinguishing hallmarks of different vessel categories are their different perivascular mural cell supports, their typical dimension, and their particular blood flow velocities. While arteries are surrounded by a multilayered coat of α-smooth muscle actin expressing mural cells (smooth muscle cells), veins are sparsely covered by smooth muscle cells as well as a distinct type of mural cell, the pericyte.

Pericytes also support capillaries (Bergers and Song 2005). Blood flow is about ten times faster in arteries than in veins and capillaries (Wayland and Johnson 1967; Ma et al. 1974). Moreover, veins, but not capillaries or arteries, are equipped with valves to prevent backflow of the blood. Endothelial cell junctions are essential in regulating the exchange between the blood and the surrounding tissue. Endothelial cell-specific adherens junctions can dissolve to permit extravasation of blood components, while tight junctions provide a persistent barrier in a vessel- and organ-type-specific manner (Dejana et al. 2001).

The lymphatics are organized in capillaries which drain unidirectionally into larger, collecting vessels. The collecting vessels bring the lymph to a sentinel lymph node from which it is carried further to eventually be drained into the subclavian vein. Lymphatic capillaries are blind-ended tubes that open up with increased interstitial pressure. The increased pressure acts to open up the lymphatic capillary by pulling on filaments anchoring the capillary to the surrounding connective tissue (Stacker et al. 2014). Collecting vessels are surrounded by an incomplete basement membrane and a thin layer of smooth muscle cells that contract and relax to propagate the lymph. Lymphatic valves in the collecting vessels prevent backflow of the lymph. Although blood and lymphatic endothelial cells are morphologically similar and share several specialized functions, they also have distinct features such as unique molecular expression patterns. This is particularly noticeable when studying endothelial cells of blood and lymphatic origin in vivo, compared to cultured cells (Wick et al. 2007). Lymphatic endothelial cells in different tissues have distinct developmental origin, but whether this reflects unique functions is not known (Potente and Makinen 2017).

Vascular Endothelial Growth Factors (VEGFs) and Their Receptors

The polypeptide growth factor VEGF was initially denoted vascular permeability factor (VPF) implying its essential role in regulation of the vascular barrier (Senger et al. 1983). The originally discovered VEGF, now denoted VEGFA, was subsequently found to be a member of a larger family of related factors. The VEGF family consists of five mammalian factors, VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor (PlGF). Of these, genetic inactivation of VEGFA and VEGFC in the mouse results in embryonic death due to defects in the development of blood vessels and lymphatics vessels, respectively. Another important feature of VEGFA is that its expression is regulated by the oxygen tension in the tissue (Ferrara 2005).

In addition, several nonmammalian VEGF-related molecules, denoted VEGFE, VEGFF, and VEGFG, have been described (Shibuya 2011). Structurally, the VEGFs are homodimeric polypeptides arranged in an antiparallel fashion, presenting one receptor-binding domains at each "pole" of the dimer (Wiesmann et al. 1997).

The VEGFs bind to three different but structurally related receptor tyrosine kinases denoted VEGF receptors 1–3 (VEGFR1, VEGFR2, VEGFR3). Although their expression patterns are not exclusively restricted to the vasculature, VEGFR2 is preferentially expressed on blood vascular endothelial cells, while VEGFR3 is primarily expressed on lymphatic endothelial cells. However, VEGFR2 is also expressed on lymphatic endothelial cells, and VEGFR3 expression is induced in newly formed vessels during angiogenesis. VEGFR1 is more broadly expressed also on a range of non-endothelial cells and is essential in regulating the motility of leukocytes. However, much less is known about VEGFR1 than the other VEGF receptors, due to the poor kinase activation of VEGFR1 in response to VEGF and due to lack of good reagents such as highly specific antibodies. Thus, the role of VEGFR1 is more unclear although there are indications that it serves primarily as a negative regulator of VEGFR2. For a detailed review on VEGF receptors, see Koch et al. (2011).

VEGFA, VEGFB, and PlGF exist as alternative splice variants which regulate their interactions with heparan sulfate and other co-receptors such as the neuropilins (Vempati et al. 2014). Co-receptors are molecules that lack intrinsic

enzymatic activity, which bind VEGF family members and sometimes also the VEGF receptors, thereby stabilizing the ligand-receptor complex, prolonging its activity. Possibly co-receptors may also influence the folding of the tertiary structure of the ligands or receptors, thereby modulating the downstream signaling. Moreover, co-receptors may have other unique functions. Several splice variants of VEGFA, denoted VEGFA121, VEGFA165, and VEGFA189 (numbers indicating the number of amino acid residues in the splice variant), have been shown to differently interact with the VEGF co-receptors and, therefore, to induce different biological responses. Thus, expression of VEGFA120 (mouse numbering) alone results in delayed outgrowth and abnormal patterning of the retinal vascular plexus compared to the wild-type condition (Stalmans et al. 2002). VEGFC and VEGFD on the other hand undergo proteolytic processing, regulating interactions with the VEGF receptors (Vaahtomeri et al. 2017).

In conclusion, none of the VEGFRs bind all the VEGF family members. VEGFR1 binds VEGFA, VEGFB, and PlGF. VEGFR2 binds VEGFA and processed VEGFC and VEGFD. VEGFR3 binds both processed and mature VEGFC and VEGFD.

Binding of VEGF to its VEGF receptor leads to receptor dimerization, activation of the intracellular tyrosine kinase activity, and tyrosine phosphorylation of both the receptor itself and of intracellular substrates for the kinase, so-called signal transducers. Through transient molecular interactions between the phosphorylated receptor and the signal transducers, mediated through their relatively specific binding motifs such as Src homology 2 (SH2) domains that recognize phosphorylated tyrosine residues, signal transduction chains are created. Through such signal transduction chains, signals can be propagated from the activated receptor to different compartments in the cell, to eventually result in a cellular response. A number of phosphorylation sites in VEGFR2 have been identified (Matsumoto et al. 2005). Several of these phosphorylation sites have been studied in loss-of-function analyses by phenylalanine knock-in, in vitro using transfected cell lines or in vivo, using recombinant mice (Sakurai et al. 2005; Li et al. 2016). The Y949 site in the VEGFR2 kinase insert is critical in regulation of vascular leakage. It serves as a binding site for the SH2 domain of T cell-specific adaptor (TSAd), which uses its proline (P)-rich domain to bind to the cytoplasmic tyrosine kinase c-Src (Sun et al. 2012). c-Src is then translocated to endothelial cell junctions where it phosphorylates the adherens junction-specific protein vascular endothelial (VE)-cadherin (see Fig. 1). Other pathways involving p21-activated kinase (PAK) phosphorylation of VE-cadherin on serine residues have also been identified (Gavard and Gutkind 2006). Interestingly, at least in vitro, VEGFR2-dependent signal transduction is suppressed by intact adherens junctions and augmented when VE-cadherin's homophilic interactions are interrupted (Lampugnani et al. 2006).

VEGFR2's enzymatic activity can be induced by shear stress exerted by blood flow, in the apparent absence of ligand (Jin et al. 2003). Induction of VEGFR2 activity may be dependent on c-Src activity which also is induced by flow (Jalali et al. 1998). Whether the flow-activated VEGFR2 transduces a full downstream signaling effect as compared with the ligand-activated receptor is unclear. Together with platelet endothelial cell adhesion molecule-1 (PECAM-1) and c-Src, VEGFR2 forms a mechanosensing complex (Tzima et al. 2005). Engagement in such complexes may be a prerequisite for both VEGFR2 and c-Src to be activated by flow.

Replacement of tyrosine (Y) at position 949 for phenylalanine, thus preventing phosphorylation and downstream signal transduction, does not interfere with normal mouse development, but it renders endothelial junctions unresponsive to VEGFA (Li et al. 2016). In the wild-type, normal condition, exposure of endothelial cells to VEGFA results in increased leakage of solvent and molecules. In contrast, in a mouse expressing $Vegfr2^{Y949F/Y949F}$, the receptor is unable to couple to TSAd and relocate c-Src to endothelial cell junctions when exposed to VEGFA. The junctions remain closed, and there is no leakage of solvent or molecules (Li et al. 2016). *Tsad* gene inactivation, globally or specifically in endothelial cells, also makes endothelial junctions unresponsive to

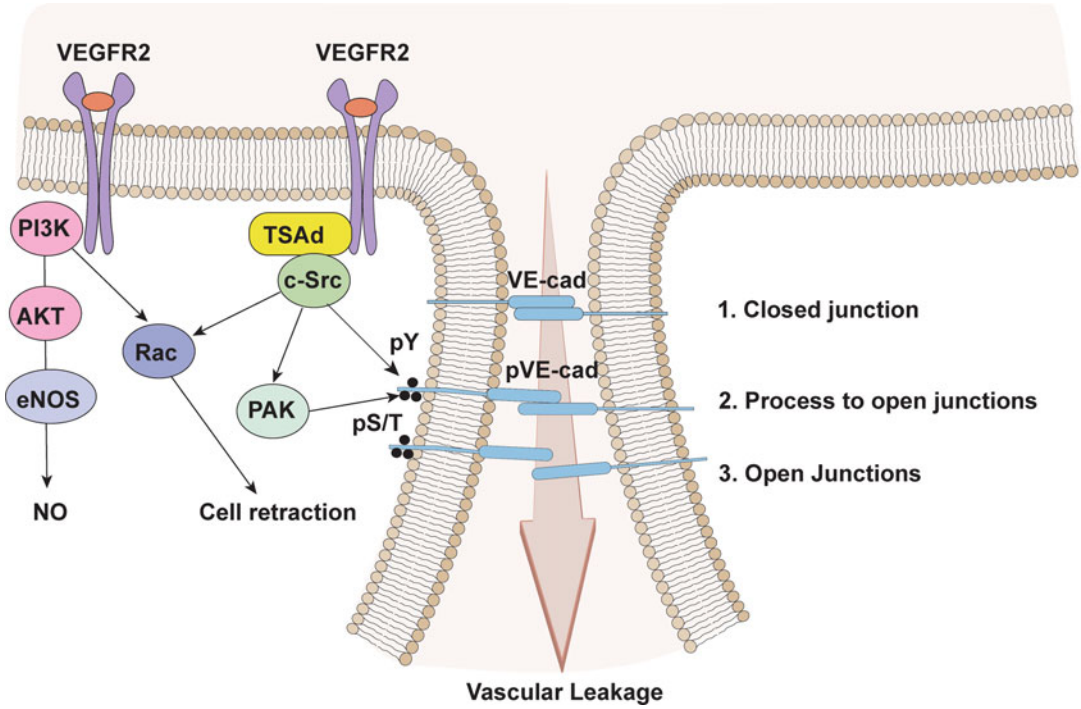

Fig. 1 Signal transduction regulating VEGFA-induced vascular leakage. VEGFR2 expressed on the surface of blood vascular endothelial cells becomes activated when binding VEGFA, resulting in induction of at least two main signal transduction chains, promoting opening of adherens junctions. One involves binding of TSAd/SRC, leading to increased tyrosine phosphorylation of VE-cadherin (VE-cad), interrupting its homophilic interactions. In another chain, PI3K promotes activation of AKT, leading to phosphorylation of eNOS and production of NO. PI3K also promotes activation of Rac which has multifaceted effects via the cell cytoskeleton leading to cell retraction. SRC can also regulate activation of PAK, leading to serine phosphorylation of VE-cadherin. For details, see the text. (*TSAd* T cell-specific adaptor, *PI3K* phosphoinositide 3'kinase, *eNOS* endothelial nitric oxide synthase, *PAK* p21-activated kinase)

VEGFA, resulting in loss of VEGF-induced vascular leakage (Sun et al. 2012).

Several studies from other laboratories implicate c-Src in phosphorylation of VE-cadherin (Weis et al. 2004; Eliceiri et al. 1999). According to the model, c-Src-induced phosphorylation of VE-cadherin disrupts VE-cadherin contacts between adjacent endothelial cells, followed by internalization and degradation or recycling of VE-cadherin (Fukuhra et al. 2006). c-Src may act to open adherens junctions not only by directly phosphorylating VE-cadherin. In a parallel signal transduction pathway, c-Src phosphorylates and activates focal adhesion kinase (FAK), which acts to anchor the actin cytoskeleton to focal adhesion sites. Focal adhesion sites are hotspots where integrin molecules in the plasma membrane bind specific extracellular matrix proteins, thereby anchoring the cell to the underlying substratum. The tension induced through the change in cell-matrix adhesion may pull on actin filaments, mediating retraction of the cell body, and pulling junctions apart.

The other VEGFR2 phosphorylation sites induce signaling pathways that may also contribute to vascular permeability regulation although this has not been directly addressed. These sites include Y1173 (Y1175 in the human VEGFR2) and Y1212 (Y1214 in the human VEGFR2). Phosphorylated Y1175 binds phospholipase C (Sakurai et al. 2005), as well as the SH2 domain molecule Shb (Funa et al. 2009). Potential binding partners for phosphorylated Y1212 have been less extensively studied. For details on their

downstream pathways, the reader is referred to Koch et al. (2011).

Whether other growth factors for which there are receptors on endothelial cells, such as PlGF (binding exclusively to VEGFR1), VEGFC/VEGFD (binding exclusively to VEGFR3), or fibroblast growth factors (FGFs, binding to FGFR1 and FGFR2), mediate acute or chronic vascular permeability has not yet been addressed in detail. A key question is whether effects are directly transduced by these factors or whether it is indirect and dependent on elevated production of VEGFA.

The angiopoietin receptor, Tie2, exerts negative regulation of VEGFA-induced vascular leakage in response to its ligand angiopoietin-1 (Ang1) (Brindle et al. 2006), through complex biology. One important effect of Ang1/Tie2 is to attract pericytes to increase the vascular support (Thurston et al. 1999). Ang1 may also stabilize junctions by promoting the recruitment of Tie2 to junctions (Saharinen et al. 2008).

The related Ang2 on the other hand may cause vessel disintegration resulting in loss of vascular integrity and massive vascular leakage, independent of VEGFA. Ang2 exerts antagonistic effect on vascular integrity in a manner dependent on Tie1. When Tie1 is lowly expressed or cleaved (Korhonen et al. 2016; Kim et al. 2016), Ang2 can act as a Tie2 agonist, rather than an antagonist. Other factors acting independently of VEGFA include inflammatory cytokines such as histamine and bradykinin, which are potent mediators of vascular leakage in inflammation. Histamine is produced by mast cells and binds to G-protein-coupled H1 and H2 histamine receptors (GPCRs) on endothelial cells (Marshall 1984). Bradykinin is cleaved from kininogen; it acts via GPCRs B1 and B2 (Sharma and Al-Dhalmawi 2003). Although other mechanisms have not been excluded, it is quite well established that exposure of vessels to either histamine or bradykinin results in activation of endothelial nitric oxide synthase (eNOS) and consequent production of NO which acts to relax vascular smooth muscle cells (Fig. 1). The relaxation results in a reduced vasotone, i.e., a widening of the vessel lumen.

The eNOS-NO pathway is strongly implicated also in VEGFA-regulated vascular leakage since ablation of eNOS expression attenuates responsiveness to VEGFA (Fukumura et al. 2001). NO may act directly on VE-cadherin to regulate its phosphorylation status, at least in vitro (Di Lorenzo et al. 2009). Another effect of NO that may affect vessel leakage is its S-nitrosylation of beta-catenin that will cause beta-catenin to dissociate from VE-cadherin, triggering the disassembly of adherens junctions (Thibeault et al. 2010).

Features Regulating Vessel Integrity

Basal Permeability

The CNS vascular barrier is guarded by specialized junctions that are impermeable in the healthy condition (see further below). In contrast, in non-CNS organs, there is a continuous basal vascular sieving (i.e., permeability) of solvent and small molecules from blood into tissues, which occurs in an "unstimulated" setting, i.e., in the absence of an elevated production of factors that promote leakage of blood constituents, such as VEGF (see below). Plasma proteins smaller than 40 kDa may extravasate spontaneously (Egawa et al. 2013), in a manner dictated by the glycocalyx (see below, "The Glycocalyx"), whereas leakage of larger molecules is restricted in a size-dependent manner. Passage of cells does not occur in the resting, unstimulated vasculature.

The mass of plasma solvent and solvents that crosses the vascular wall depends on at least three different factors as described previously (Dvorak 2010): (1) hemodynamic forces, i.e., blood pressure and blood flow, (2) concentration gradients of molecules and vascular area available for exchange, and (3) the intrinsic permeability of the vascular wall. Of note, solvent may also leak from tissues into the blood, dependent on these parameters.

In peripheral organs, blood vessels of all types may allow basal vascular permeability; however, it is probable that it is less prevalent in arteries as their intrinsic permeability is lower. The constant

sieving of solvent and small molecules is important in maintaining the interstitial pressure in the tissue. It also serves to maintain the immune surveillance function of the lymphatics as interstitial fluid collected by the lymphatics is carried via lymphatic capillaries to lymph nodes where foreign antigens will be exposed to the immune system (Cueni and Detmar 2008).

Whether lymphatic vessels also show a continuous basal sieving of solvent and small molecules has not been directly addressed, but it is likely to occur. In pathologies, lymphatic endothelial junctions become "leaky" in an Ang2-dependent manner, leading to changes in VE-cadherin phosphorylation (Zheng et al. 2014).

Endothelial Fenestrations

Endothelial cells in many vessels form an uninterrupted vasculature. In certain organs, however, the endothelial cells display specialized structures to facilitate rapid transport across the endothelium. See Tse and Stan (2010) for a detailed description. One example is the fenestrated endothelium that is present in vessels in endocrine glands, digestive tract mucosa, and kidney peritubular capillaries. Fenestrations are regions where the apical and basolateral endothelial membranes are fused to create circular pores that may be covered by a diaphragm. A key protein in the diaphragm is plasmalemmal vesicle protein-1 (PV1), organized in radial fibrils. Loss of PV1 does not prevent formation of fenestrae as such but results in loss of the diaphragm and severe leakage of plasma proteins (Stan et al. 2012).

There are naturally occurring fenestrae, or gaps, without diaphragm, i.e., in the kidney glomerulus (Tse and Stan 2010). The sinusoidal endothelium in the liver and the bone marrow also presents large gaps without a diaphragm. These gaps are heterogeneous but of larger diameter than the endocrine vessel fenestrae. Signaling through the actin cytoskeleton has been shown to regulate the diameter of these openings and thereby regulate vascular barrier function (Venkatraman and Tucker-Kellogg 2013; Braet et al. 1995).

The Glycocalyx

The glycocalyx is a carbohydrate-rich layer lining the vascular endothelium which long escaped detailed studies as it often was lost during fixation procedures in preparation for microscopy; moreover, endothelial cells in culture do not form a glycocalyx. While its exact composition has not been defined, the glycocalyx consists of a membrane-bound mesh of proteoglycans, glycoproteins, and glycosaminoglycans, which along with trapped plasma proteins and soluble glycosaminoglycans form an extensive three-dimensional structure extending into the vessel lumen. Rather than being static, the glycocalyx components are continuously turned over (Reitsma et al. 2007). The glycocalyx is vulnerable to insults such as inflammation, trauma, and hemorrhagic shock, which leads to exposure of the underlying endothelium to the insult. The glycocalyx influences mechanotransduction, hemostasis, and blood cell-vessel wall interactions. In particular, the glycocalyx is an important determinant in vascular permeability and selectivity properties of the vascular wall. Thus, the glycocalyx forms the principal molecular sieve at the endothelial wall, where the spacing between fibers in the glycocalyx allows penetration of molecules up to the size of albumin (Curry 2005).

The Blood-Brain Barrier (BBB)

The BBB is a unique barrier with the purpose of preventing the brain from exposure to the blood and the adverse consequence of edema, which may be detrimental for the tightly enclosed brain. The brain vasculature has, in addition to adherens junctions, also high resistance tight junctions and an abundant basement membrane. Perivascular components such as astrocytes, pericytes, and neurons participate functionally in creating the BBB (Paolinelli et al. 2011). A unique feature of the BBB is the transendothelial vesicular transport of a range of nutrients and metabolic waste products (Strazielle and Ghersi-Egea 2013). There is a keen interest from the pharmaceutical industry to find strategies to interrupt the BBB for

drug delivery. There is still limited information on to what extent the BBB can be transiently opened in response to growth factors and inflammatory cytokines (Hudson et al. 2014).

The Vesiculo-Vacuolar Organelle (VVO)

Based on the use of various tracers, for example, electron-dense ferritin, VVOs have been implicated as a possible pathway for macromolecular extravasation (Kohn et al. 1992). The VVO has been described and interpreted using transmission electron microscopy analyses, which have shown that VVOs are prominent structures in both tumor-supplying and normal vessel endothelial cells (Dvorak and Feng 2001).

There is general consensus on the notion that vesicular transport across the endothelium (transcytosis) is an important mechanism for delivery of macromolecules to tissues, in particular in the CNS. During transcytosis, caveolae, specialized regions in the plasma membrane (PM), "pinch off" from the PM to form discrete vesicular carriers that shuttle to the opposite side of the endothelium where vesicles fuse with the PM and discharge their cargo into the perivascular space. Endothelial transcytosis may occur in specialized vascular beds or under particular physiological conditions. Transcytosis has been described in the brain vasculature, and it is elevated under conditions when the BBB is disrupted due to pericyte deficiency (Armulik et al. 2010). VVOs may be one possible mechanism for transcytosis.

Vesicles and vacuoles that make up the VVO were originally thought to derive from caveolae. A main protein in caveolae is caveolin-1. While caveolin-1 knockout mice lacked caveolae and showed reduced permeability to macromolecules, the vasculature still contained VVOs (Chang et al. 2009). The exact composition of the VVO is therefore presently not known. A challenge in further analyses of VVOs is that they cannot be detected by conventional light microscopy. Moreover, there is at present no genetic loss-of-function model to study VVOs.

Endothelial Junctions in Lymphatic and Blood Vessels

Endothelial junctions play an important role in the regulation of passage of solvent, molecules, and cells across the vessel wall. In most organs, the endothelial cells form a dynamic barrier between the blood and the tissue. In resting conditions, the vasculature continuously leaks solvent and small molecules (basal sieving; see "Basal Permeability"), but restricts extravasation of larger molecules and cells. In many diseases, including cancer and chronic inflammatory conditions, the vascular barrier disintegrates, and leakage increases and may become chronic. The leakage of larger molecules and cells results in edema, inflammation, and, often, disease progression (Nagy et al. 2008).

In blood vessels, endothelial junctions consist of tight junctions and adherens junctions. Both types of junctions express proteins unique for blood endothelial cells as well as common junction proteins seen also in epithelial cell-cell junctions. Claudin-5 is preferentially although not uniquely expressed in endothelial cells. In the CNS, Claudin-5 has a critical function in maintaining the BBB (Argaw et al. 2009). In contrast, other tight junction proteins such as zona occludens1 (ZO1; also denoted tight junction protein-1) are more broadly expressed in endothelial and epithelial cells. There is still incomplete understanding of the composition of the endothelial tight junction, which may vary between different types of endothelial cells in arteries, capillaries, and veins and also between different vascular beds such as in the CNS and in peripheral organs. It is also unclear to which extent the tight junction barrier can be regulated by exogenous factors, i.e., made more or less stringent. There appears to be a molecular communication between adherens junctions and tight junctions, for example, via VE-cadherin and ZO1 in vitro (Tornavaca et al. 2015), but it remains to be shown that this communication occurs also in vivo.

The main component of the endothelial-specific adherens junction is VE-cadherin (Dejana et al. 1999). In contrast to tight junctions, adherens junctions can be induced to dissolve in

a specific and transient (in physiology) or chronic (in disease) manner, allowing leakage to occur (see below). The dissolution involves interruption of homophilic interactions between VE-cadherin molecules on opposing endothelial cells, followed by internalization of VE-cadherin. There are several VE-cadherin-associated molecules of critical importance for adherens junction maturation and stability: (1) p120-catenin which connects VE-cadherin to members of the Rho GTPase family (Kourtidis et al. 2013), (2) alpha-catenin which connects VE-cadherin with the actin cytoskeleton via binding to p120 and beta-catenin (Brieher and Yap 2013), (3) beta-catenin which when released from the VE-cadherin complex can act as a transcriptional regulator through the Wnt/Frizzled family of ligands and receptors (Valenta et al. 2012), and (4) plakoglobin (also denoted gamma-catenin). For details, see Dejana et al. (2008).

As mentioned above, the vasculature in the CNS is equipped with a particular strong barrier, the blood-brain barrier (BBB) (Paolinelli et al. 2011), to protect the brain parenchyma from detrimental edema. The detailed composition of the specialized tight junctions protecting the CNS vasculature is not yet known.

Junctions between lymphatic endothelial cells vary in morphology and function dependent on the vessel type (Baluk et al. 2007). Lymphatic capillary cell-cell contacts are denoted button junctions based on their discontinuous, oak-leaf morphology. They are considered leaky and can open up by mechanical pulling on lymphatic ligaments that extend radially from the lymphatic capillary (Leak and Burke 1966). Junctions in the collecting lymphatics are denoted zipper junctions, which are continuous junctions of higher integrity. There are also intermediary/transitory junctions in between the capillaries and collecting lymphatic vessels. While lymphatic endothelial cells also express VE-cadherin, as well as a range of tight junction molecules, it is unclear whether lymphatic junctions can be dissolved transiently in a similar manner to adherens junctions in blood vessels.

Adherens junctions dissolve in response to a number of stimuli; while the focus here is on VEGFA, inflammatory cytokines and other factors can also induce dissolution of endothelial adherens junctions. The causative factor may depend on the organ and particular biology, e.g., inflammation vs tissue growth. VEGFA causes a rift in VE-cadherin homophilic interactions, bridging between adjacent endothelial cells (Fig. 1). The rift is induced through a triggering signaling that involves hyperphosphorylation of VE-cadherin. However, in vivo, VE-cadherin is phosphorylated also in the basal, unstimulated state (Orsenigo et al. 2012; Li et al. 2016), possibly through flow-mediated activation of c-Src, which triggers VE-cadherin phosphorylation directly or indirectly (Fig. 1). The additional event induced by VEGFA causing VE-cadherin internalization remains to be identified, but may involve enhanced VE-cadherin internalization or particular intracellular trafficking. Mechanisms different from a direct phosphorylation of VE-cadherin by c-Src have been suggested (Gavard and Gutkind 2006).

VE-cadherin is phosphorylated on at least three tyrosine residues, Y658, Y685, and Y731, which are differently engaged in regulating junctional passage of molecules and cells. Phosphorylated Y685 is required for VEGFA-induced junctional leakage, whereas phosphorylated Y731 is required for passage of inflammatory cells, as deduced from studies of mice lacking individual phosphorylation sites (Wessel et al. 2014). The role of phosphorylated Y658 appears to be related to that of the pY685 site as they are regulated in a similar manner (Orsenigo et al. 2012).

When VEGFA is administered to the healthy tissue, the dissolution of adherens junctions is transient and the junctions will soon close again in part due to VE-cadherin recycling and reappearance on the cell surface (Fukuhra et al. 2006). Using transmission or scanning EM, junctions have been captured in their open state, revealing the kinetics of opening and closure in vivo (Baluk et al. 1997). In diseases characterized by excess vascular leakage, the regulation of junction dynamics is lost and the junctions remain open. This is denoted chronic permeability/leakage (Nagy et al. 2008); see further below.

Other mechanisms that may prevail in regulating junctional integrity in response to VEGFA include the rearrangement of the actin cytoskeleton in a manner that may involve c-Src-mediated activation of small GTPases such as Rac (Fig. 1). Phosphoinositide 3′ kinase (PI3K) activation in response to VEGFA may also orchestrate activation of small GTPases. Retraction of the endothelial cell body involving cytoskeletal rearrangements has been implicated in mediating increased vascular permeability (Majno et al. 1969). Thus, the action of intracellular motor proteins causes cells to contract in a manner that facilitates opening of paracellular junctions. However, the cell retraction hypothesis has been challenged, and the cell shape changes observed have been attributed to a natural recoil process occurring when cell-cell junctions are disassembled (Adamson et al. 2003; Waschke et al. 2004). The role of the actin cytoskeleton needs to be further studied.

Blood Flow Regulation: Implication for Basal Sieving

The basal rate of blood flow, most often estimated from the movement of erythrocytes, is an essential parameter dictating the rate of exchange across the vessel wall (Baskurt et al. 2004; Meininger and Davis 1992). Thus, with increased local blood flow, the basal sieving increases in the permissive vasculature. Blood flow is influenced by a number of parameters such as (1) the type of blood vessel including its mural support and its diameter, (2) the blood volume and its viscosity, and (3) the blood pressure, regulated by the renin-angiotensin II-aldosterone axis and influenced by the elasticity of the vessel wall and the tortuosity and branching of the vessel. A range of factors regulate the local blood flow by affecting the vessel diameter, hence its tone, through constriction or dilation of the arteriolar mural cell coat (Bergers and Song 2005). ATP, angiotensin II, endothelin, and adrenalin all induce vasoconstriction. Dependent on the context, adrenalin can also induce vasodilation. Other factors inducing vessel dilation include adenosine, prostaglandins, and NO. NO is regarded as an essential regulator of vascular permeability as well as vascular leakage, in response to inflammatory cytokines and VEGFA.

NO is produced from arginine by the enzyme eNOS (also denoted NOS3), which belongs to a family of three NOS members, eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS). Both eNOS and iNOS are expressed in endothelial cells. While it is clear that eNOS exerts an important function in the vasculature, it has not been shown whether iNOS has a similar role. VEGFA and inflammatory cytokines activate eNOS through phosphorylation by the serine/threonine kinase AKT (Phung et al. 2006; Fulton et al. 1999; Fukumura et al. 2001; Dimmeler et al. 1999) (see Fig. 1). AKT is not the only serine/threonine kinase that can phosphorylate and activate eNOS, but it is the best studied pathway. NO is a potent regulator of the vascular tone; it mediates vasodilation by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells, which causes their relaxation (Forstermann and Sessa 2012).

Local regulation of blood flow is moreover thought to be controlled through precapillary sphincters. In the mesentery, precapillary smooth muscle sphincters have been described, consisting of folds of smooth muscle cells, arranged concentrically and distinct from the perineural coat, located at the point where a capillary branch leaves an arteriola. Based on electron microscopy analyses and a thorough investigation of the literature, Sakai and Hosoyamada concluded that precapillary sphincters are missing from a wide range of other capillary beds (Sakai and Hosoyamada 2013). Thus, how arteriolar resistance is exerted is still a matter of debate.

VEGF-Induced Transient Vessel Leakage

The vasculature is protected from uncontrolled leakage in different manners dependent on the vessel type and anatomical location. Thus, large molecules and cells require an active disruption of the vascular barrier in order to extravasate to the surrounding tissue. Such induced leakage takes

place in peripheral (non-CNS) organs preferentially in postcapillary venules (Majno et al. 1969; Kohn et al. 1992), but capillaries and larger venules may also leak (Roberts and Palade 1995). In contrast, arteries and arterioles do not leak. Overall, studies on the regulation of vascular leakage often suffer from the lack of physiological readouts, that is, instead of following leakage of endogenous substances, various tracers are followed that may or may not be representative of physiological leakage. It is clear, however, that leakage of molecules and cells to some extent is differently regulated.

Leakage of Molecules

Plasma contains three main molecular constituents: albumin, globulins, and fibrinogen (Adkins et al. 2002). Extravasation of macromolecules serves diverse purposes, for example, to maintain the balanced blood and interstitial pressures and to carry other molecules, such as hormones and lipids, across the vessel wall. Extravasated fibrinogen, processed to fibrin, may form a provisional matrix on which new blood vessels extend (Dvorak et al. 1987). Extravasated plasma molecules in peripheral tissues are believed to preferentially pass through opened endothelial junctions.

Leakage of Cells

Junctional gaps appear to be required also for extravasation of inflammatory cells; however, the preferred route of exit for leukocytes and immune cells has been difficult to unequivocally sort out (Vestweber et al. 2014). Inflammatory cells adhere to the endothelium through binding to specific adhesion molecules on the endothelial surface. The cells can then transmigrate directly through the thin endothelial wall or through endothelial junctions (Vestweber 2012; Phillipson and Kubes 2011; Nourshargh et al. 2010). The route of choice might depend on the stimulus, type of leukocyte, and vascular bed. Interestingly, expression of a fusion protein between VE-cadherin and α-catenin in mice resulted in a complete sealing of junctions to macromolecular extravasation (Schulte et al. 2011). Inflammatory cell extravasation was however not completely restricted. Indeed, the extent of immune cell extravasation appeared not to be affected (Schulte et al. 2011). It is possible that different inflammatory cells extravasate through different mechanisms or that the cells are sufficiently plastic to adopt to the possibilities offered in the particular situation. Finally, exit of inflammatory cells may be differently regulated in acute and chronic inflammation.

Vascular Leakage in Disease

Vascular Leakage and Cancer

The tumor vasculature displays a spectrum of morphological and functional abnormalities including loss of vessel hierarchy, increased tortuosity, poor perfusion, instability, and increased vascular leakage (McDonald and Baluk 2005). To a considerable extent, the tumor vessel phenotype is a consequence of hypoxia-driven persistent VEGFA production (Liao and Johnson 2007). Anti-angiogenic treatment, e.g., using VEGFA-blocking antibodies or VEGFR kinase inhibitors, therefore induces a more normal tumor vessel morphology and attenuates the exaggerated permeability (Jain 2005). The therapeutic benefit of anti-angiogenic treatment in prolonging progression-free and overall survival depends on the cancer diagnosis. The reader is referred to in-depth recent reviews on this important matter; see, e.g., Singh and Ferrara (2012). To what extent the potential benefit of anti-angiogenic therapy on growth of the primary tumor and suppression of metastatic spread primarily depends on suppression of vascular leakiness or whether other effects of the treatment, e.g., on neoangiogenesis in the tumor, are more important is very challenging to distinguish.

The excess vascular leakage in cancer has a range of deteriorating effects on the microenvironment of the tumor including increased interstitial pressure leading to impaired therapeutic delivery (Azzi et al. 2013). Moreover, the leaky vasculature may facilitate both leukocyte infiltration into the tumor and escape of tumor cells into the blood to establish distant metastases.

Vascular Leakage and Myocardial Pathology

Tissue damage in myocardial infarction (MI) is triggered by tissue ischemia as a consequence of vessel occlusion and poor blood flow. This in turn leads to induction of VEGFA production and an acute increase in vascular leakage and a consequent tissue edema, impairing the ability of the heart to pump efficiently. Moreover, the increased vessel leakage is manifested as increased infiltration of inflammatory cells in the acute phase after vessel occlusion (Nagy et al. 2008; Weis 2008). One of the first cell types to enter the infarcted myocardium is the neutrophil (Carbone et al. 2013). Neutrophils contribute to tissue damage, e.g., by producing several enzymes that produce reactive oxygen species (ROS) and other tissue-damaging metabolites such as nitrosylated products. Such enzymes include nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and myeloperoxidase (MPO) (Carbone et al. 2013). Elevated MPO levels predict the risk of heart disease in subgroups otherwise associated with low risk (Meuwese et al. 2007; Karakas et al. 2012). Elevated MPO levels also independently predict the early risk of future cardiovascular events in patients with acute coronary syndromes (Baldus et al. 2003; Cavusoglu et al. 2007).

Vascular Leakage in Ocular Diseases

The vasculature in the eye is protected by the blood-retinal barrier (BRB), which is maintained by tight junctions between retinal capillary endothelial (RCE) cells and retinal pigment epithelial (RPE) cells, which form the inner and outer BRB, respectively (Barar et al. 2009). RCE cells possess intercellular tight junctions, which are formed by RCE and glial cells (Gardner et al. 1999). Loss of normal BRB function is a common feature to many retinal degenerative disorders including age-related macular degeneration, diabetic retinopathy, and retinal vein occlusions (Stewart 2012). Age-related macular degeneration patients present focal ischemia in the outer retina with associated inflammation, which induces VEGF production and angiogenesis resulting in vessel leakage. Prolonged elevation of blood sugar concentrations in diabetic patients causes endothelial apoptosis, basement membrane thickening, and pericyte loss, accompanied by increased VEGF production and transvessel passage. Retinal vein occlusions can be attributed to hemodynamic disturbance (increased coagulation, impaired flow properties) resulting in ischemia and increased VEGF synthesis (see Stewart 2012 for details). The common aspects of many eye diseases are therefore ischemia, increased VEGF production, and vascular leakage (Miller et al. 2013). The excess leakage has been attributed both to the overstimulated, abnormal vasculature and to changes in the phosphorylation of tight junction proteins such as occludin and ZO1 (Antonetti et al. 1999).

Lymphatic Neoangiogenesis and Cancer

Lymphatic drainage in the healthy tissue is regulated by the interstitial pressure, opening up the lymphatic capillaries, and possibly by signaling in the blood vasculature resulting in release of cytokines regulating signaling in the lymphatic endothelium (Fig. 2). In cancer, drainage is inadequate in spite of the elevated interstitial pressure and elevated signaling in the blood endothelium, possibly due to the persistent and dysregulated nature of the signaling. Moreover, cancer lymphatic vessels are often collapsed due to the excessive interstitial pressure further exacerbating the edema (Stacker et al. 2014). Several cell types in the cancer produce lymphatic growth factors, including VEGFC that binds and activates VEGFR3 (Adams and Alitalo 2007). Similar to the overstimulated and dysfunctional blood vasculature, the lymphatics may undergo neoangiogenesis in cancer, which would facilitate draining of the tumor edema on the one hand but also provide a route for spread of the cancer via the lymphatics. However, the relationship

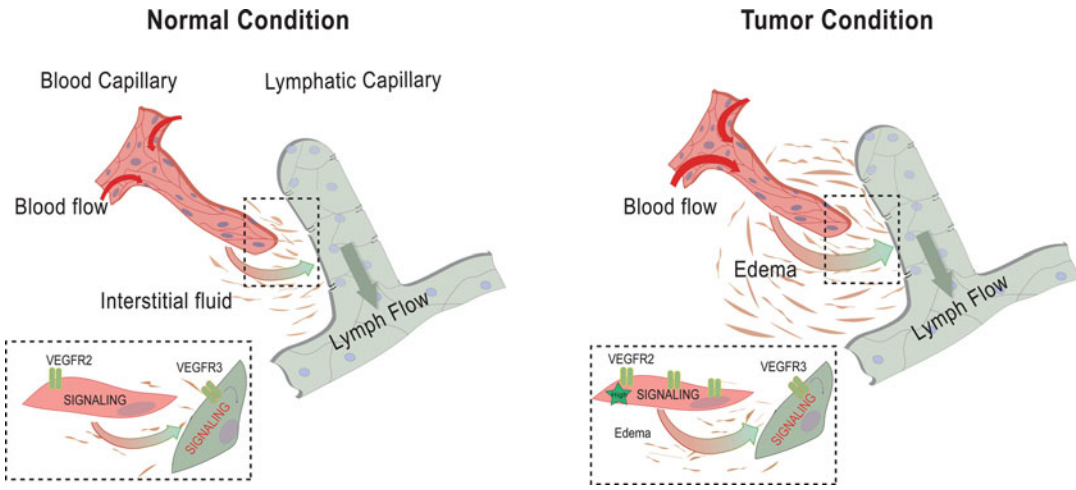

Fig. 2 Communication between the blood and lymphatic vasculature in normal and tumor conditions. Interstitial fluid accumulates as a consequence of basal sieving from the blood vasculature and is drained by the lymphatics (left part of the panel). Endothelium in blood and lymphatic vessels may also communicate by VEGFA/VEGFR2 signaling resulting in production of factors regulating lymphatic endothelial signaling (boxed to the left). In cancer (right part of the panel), excessive VEGFA/VEGFR2 signaling leads to elevated and chronic vascular leakage and increased interstitial accumulation of fluid. Due to poor drainage by the lymphatics, edema builds up. The lymphatic flow is impaired due to collapse of the lymphatic vessel and possibly through exaggerated and dysregulated signaling (indicated by star) from the blood endothelium communicating with the lymphatic endothelium (boxed to the lower right)

between formation of new lymphatic vessels and metastatic spread in cancer is as yet incompletely understood.

Imaging Vascular Flow and Integrity

Recent advances in microscopy techniques combined with computational analysis have created a paradigm shift in studying vascular flow and permeability. Powerful imaging systems have been developed to monitor microvasculature dynamics in vivo, including various tomography techniques such as Doppler ultrasound, dynamic contrast-enhanced magnetic resonance imaging, and optical imaging methods (Jennings et al. 2008). Optical imaging techniques are most commonly used for non-clinical and in-depth study of vascular flow and leakage.

Fluorescent tracer dyes as well as variable-sized fluorescent probes combined with intravital microscopy provide a more detailed understanding of vascular flow and permeability under normal and diseased conditions (Fukumura et al. 2010). Upright imaging using normal epifluorescence (Pink et al. 2012) and multiphoton imaging (Egawa et al. 2013, Brown et al. 2001) are the two most common techniques of optical imaging of blood vessels. The former, i.e., wide-field microscopy, is often limited by the depth of penetration and resolution, while these limitations can be overcome using a multiphoton microscope. Near infrared imaging (NIR) using fluorescent indocyanine green and molecular probes also provides an in-depth understanding of vascular as well as lymphatic permeability under normal and tumor conditions. Conducted at near infrared wavelengths (650–900 nm), NIR has advantages of enhanced tissue penetration, decreased tissue absorption, and decreased autofluorescence (Proulx et al. 2013).

Vascular flow and permeability data obtained from the microscopy techniques combined with biophysical modeling can provide insights and predictions to flow. Such models provide useful insights to the understanding of blood flow in tumors (Soltani and Chen 2013), interstitial

pressures and metastasis (Jain et al. 2007), and transport of nanoparticle therapy (Stapleton et al. 2013). A combined approach of imaging and modeling would therefore provide an increased understanding of changes in blood flow during tumor development and could also help predict the efficacy of drug transport.

Perspectives

Excess vascular permeability resulting in edema and swelling of the tissue (in latin; *tumor*) was noted already in the encyclopedia *De Medicina* by Aulus Cornelius Celsus (25 BC–50 AD) as one of the four cardinal signs of inflammation (*tumor, rubor, calor, dolor*). A focus of interest today is whether specifically suppressing excess vascular permeability is therapeutically beneficial in a range of diseases. Thereby, tissues engaged in the disease would be less edematous, and the interstitial pressure would be lower, allowing more efficient delivery of conventional therapeutics, such as chemotherapy to treat cancer. A more efficient delivery of chemotherapeutics, perhaps at a lower, less toxic dose, is obviously of considerable interest clinically. It would be expected that the barrier presented by non-leaky vessels would provide better perfusion and thereby facilitate tissue homeostasis and promote healing.

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Cross-References

- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Imaging Tumor Angiogenesis
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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