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Disorders of Vascular Permeability

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Abstract

The endothelial barrier maintains vascular and tissue homeostasis and modulates many physiological processes, such as angiogenesis. Vascular barrier integrity can be disrupted by a variety of soluble permeability factors, and changes in barrier function can exacerbate tissue damage during disease progression. Understanding endothelial barrier function is critical for vascular homeostasis. Many of the signaling pathways promoting vascular permeability can also be triggered during disease, resulting in prolonged or uncontrolled vascular leak. It is believed that recovery of the normal vasculature requires diminishing this hyperpermeable state. Although the molecular mechanisms governing vascular leak have been studied over the last few decades, recent advances have identified new therapeutic targets that have begun to show preclinical and clinical promise. These approaches have been successfully applied to an increasing number of disease conditions. New perspectives regarding how vascular leak impacts the progression of various diseases are highlighted in this review.

Keywords

barrier; endothelium; junctions; permeability; angiogenesis

INTRODUCTION

Regulation of endothelial barrier function is critical for vascular function and integrity. Whereas single cells exchange gases and small molecules with their environment through diffusion, the vertebrate vasculature is permeable, which is important for the maintenance of normal fluid homeostasis and adequate gas exchange. Vascular permeability is a complex process and is influenced by many different variables. Factors that impact vascular permeability include (a) the intrinsic properties of the microvessels involved (e.g., capillaries and venules); (b) the size, shape, and charge of the extravasating molecules (water and solutes); (c) the anatomic pathways through which molecules cross the endothelial barrier, such as the channels, fenestrations or vesicles within individual endothelial cells

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(transcellular) or the junctional complexes between adjacent endothelial cells (paracellular); (d) the time course over which permeability is measured; and (e) the specific vascular bed that is being investigated. Whereas transport of protein and lipids via transcellular routes contributes to the maintenance of vascular homeostasis, pathological vascular leak takes place largely by paracellular routes (1).

Endothelial barrier function:

the integrity of the endothelial structure, which undergoes changes at the cytoskeleton, cell-cell junction complexes, and cell attachments to extracellular matrix and basement membrane

Transcellular:

describes the transport of solutes through a cell, including passage through both the apical membrane and basolateral membrane

Paracellular:

describes the transfer of substances across an epithelium via passage through the intercellular space

Angiogenesis is the sprouting of new capillaries from preexisting vessels. Under physiological conditions, angiogenesis is an essential biological process that satisfies the increasing metabolic demand for nutrients and oxygen in rapidly expanding tissues (2). In adults, physiological angiogenesis occurs during reproduction, tissue remodeling (such as in adipose expansion and exercise) and wound repair; under these circumstances, the new vessel growth is local and limited (3). In contrast, the neovascularization that occurs under pathological conditions generally lacks the same spatial and temporal control. Angiogenesis plays a central role in cancer and many non-neoplastic diseases, such as rheumatoid arthritis, atherosclerosis, diabetic retinopathy, and age-related macular degeneration (AMD) (4). In most of these conditions, the new vessels are tortuous, display abnormal mural cell investment, and are hyperpermeable. The leakage of plasma proteins, in turn, leads to remodeling of the extracellular matrix (ECM) and tissue damage (5).

Angiogenesis:

the physiological process through which new blood vessels form from preexisting vessels

Age-related macular degeneration (AMD):

the leading cause of severe vision loss in people over age 60, which afflicts the central macula

Thus, abnormal control of this critical function may have devastating consequences. For example, solid tumors release factors that promote the growth of disorganized and leaky blood vessel networks that display disturbed blood flow, inflammatory cell infiltration, and tumor cell extravasation (6). The processes of vascular permeability and angiogenesis are regulated by a delicate balance of polypeptide factors and inhibitors (7). This review focuses on permeability characteristics of normal and pathological microvessels and on the mechanisms thought to control permeability, and concludes with possible implications for therapeutics.

New blood vessels form by two distinct mechanisms: vasculogenesis and angiogenesis (8, 9). In vasculogenesis, endothelial cell precursors known as angioblasts associate to form a network of primitive vessels. Cords lacking lumens are first formed, and these then remodel into patent vessels via tubulogenesis (9). In angiogenesis, new blood vessels form from the existing blood vessels, generally venules, by budding and sprouting. Whereas new blood vessels that form during development do not generally appear to be leaky, vessels associated with pathologies display greatly increased permeability. Initially described in 1935, and later confirmed in 1946, permeability was demonstrated when dyes leaked out of the capillaries of the developing amphibian tail (10) and during wound healing (11). Ultrastructural studies of wound healing models revealed that intravenous injection of a large molecular weight tracer, colloidal carbon, led to its deposition in the interstitium (12).

Today, the hyperpermeable nature of neovessels is recognized as a cardinal feature of abnormal angiogenesis. Although the vast majority of studies have demonstrated an increase in vascular permeability during pathologies such as heart disease, stroke, cancer, and ocular diseases, the regulation of vascular permeability is also critical during normal physiological angiogenesis, including exercise-induced neovascularization, hair growth, wound repair, and the female reproductive cycle.

ULTRASTRUCTURAL CHANGES

Structure of Endothelial Layers

Virtually all tissues (with the exception of the cornea, the lens, the vitreous humor, and cartilage) are supplied by a vasculature that delivers nutrients and remove waste products. The majority of transport occurs across capillaries; the narrow caliber of capillaries facilitates efficient transfer of oxygen and molecules, and their branching structure provides an expanded surface area. Gases, hydrophobic molecules and small hydrophilic molecules pass through capillaries freely, whereas circulating macromolecules are unable to cross the lipid membranes and are transported across capillaries only through bulk fluid phase transfer. The first site of fluid extravasation is the endothelium, the cells that line the entire vascular tree. The endothelium—at various levels of the vascular tree and in different microvascular beds—differs in structure and function. Endothelial cells are typically elongated in shape and have a surface coat termed a glycocalyx, a negatively charged glycosaminoglycan layer (13). Endothelial cells, which are anchored to a basement membrane by integrins, can be either continuous or fenestrated, reflecting their physiological function (14).

Endothelium:

a thin layer of epithelial cells lining the insides of the blood vessels

The capillary endothelium of the central nervous system, including the retina, is the site of the blood-brain barrier, a filtering mechanism between the brain and the blood (15), which forms an effective barrier to fluid transfer (16). Extensive intercellular tight junctions between endothelial cells contribute to this, as do a very low level of transcellular vesicle transport and a continuous basement membrane, all of which restrict the movement of fluid and molecules into the tissue. Other capillary beds with a continuous endothelial layer include those of the heart, connective tissue, and muscle. In contrast, a number of capillary beds are characterized by discontinuous endothelium, including tissues whose functions are characterized by secretion, filtration, or particularly high vascular flow, such as the kidney, whose vasculature is fenestrated.

Vascular Barrier Organization

The structure of the vessels varies depending on their location in the vasculature and reflects both genetic and environmental influences. Larger vessels (arteries, arterioles, veins, and venules) consist of an endothelial cell lining with variable layers of smooth muscle cells, whereas capillaries and postcapillary venules have associated pericytes, the microvascular equivalent of smooth muscle cells. The passage of macromolecules, fluids, and cells through the endothelial barrier is affected by many different variables including the intrinsic properties of the microvessels involved (e.g., capillaries and venules); the size, shape, and charge of extravasating molecules (water and solutes); and the anatomic pathways through which molecules cross the endothelial cell barrier (transcellular versus paracellular) (Figure 1).

Transcellular transport.—Transcellular pathways involve caveolae, vesiculo-vacuolar organelles (VVOs), and fenestrae. Small molecules (less than 3 nm) are commonly transported by a transcellular route, which requires a system of trafficking vesicles—VVOs. The vesicles or vacuoles of VVOs vary in size from caveolae-sized vesicles to vacuoles with volumes as much as ten-fold larger (17). Several permeability factors such as vascular endothelial growth factor (VEGF)-A and histamine have been demonstrated to activate VVOs (18). The VVOs rely on caveolin-1 function for albumin transport, for example, which transports fatty acids, and vitamins. Of note, caveolin-1 plays a dual regulatory role in microvascular permeability in that it stabilizes caveolae structure, allowing for transcytosis, but also acts as a negative regulator through endothelial nitric oxide synthase (19).

Vesiculo-vacuolar organelles (VVOs):

endothelial cell permeability structures that provide the major route of macromolecule extravasation at sites of increased vascular permeability induced by VEGF

Fenestrae:

small pores in endothelial cells that allow for rapid exchange between blood vessels and surrounding tissue

Vascular endothelial growth factor (VEGF):

a potent angiogenic factor first described as an essential growth factor for vascular endothelial cells, upregulated in many tumors and other angiogenic diseases

Paracellular transport.—Whereas the homeostatic transport of proteins and lipids occurs largely via transcellular routes, pathological vascular leakage usually takes place by paracellular routes (1). Cells and macromolecules larger than 3 nm escape the blood vessel via the paracellular pathway, which is mediated by the coordinated opening and closing of the endothelial cell-cell junctions. The endothelial barrier is largely maintained by adherens and tight junctions. Among the adherens junction proteins, the most notable is vascular endothelial (VE)-cadherin (20). In mice, VE-cadherin gene deletion results in early embryonic lethality due to massive vascular defects, whereas loss of its function in adults provokes a hyperpermeability phenotype (21, 22). In addition, in vivo disruption of VE-cadherin with a function-blocking antibody leads to vascular leak and hemorrhage (23). Of note, a number of other adhesive proteins accumulate in or close to adherens junctions including N-cadherin, platelet-endothelial cell adhesion molecule and junctional adhesion molecules (JAMs). JAMs are believed to support leukocyte recruitment and extravasation under inflammatory conditions. They appear to promote leukocyte movement through intercellular junctions, thus playing an important role in vascular permeability and leukocyte transmigration. For a more detailed review on the role of JAMs in inflammation and vascular pathology see Reference 24.

Vascular endothelial (VE)-cadherin:

a calcium-dependent adhesive molecule, exclusively and constitutively expressed in endothelial cells

Tight junctions bind endothelial cells to one another as a monolayer, blocking diffusion along the apical and basolateral poles (25). Tight junctions are composed of transmembrane adhesion proteins, occludins and claudins, JAM family proteins, and intracellular connectors, including zona occludens (ZO)-1, 2, and 3. In contrast to VE-cadherin, deletion of the claudin-5 gene does not impair murine embryonic development but rather leads to postnatal death caused by defective blood-brain barrier (26). Thus, whereas VE-cadherin is instrumental in vascular barrier integrity, the claudins may have a more restricted role (27). Nevertheless, adherens and tight junctions functionally and structurally influence cell polarity, differentiation, survival, and the maintenance of tissue integrity.

Zona occludens (ZO):

tight junctions formed by the fusion of integral proteins of the lateral cell membranes of adjacent epithelial cells, limiting transepithelial permeability

Vascular endothelial cells are anchored to the ECM through integrin-based adhesion complexes, referred to as focal adhesions. Integrins act as a bridge between the ECM and the actomyosin cytoskeleton (28), allowing the endothelium to adapt to extracellular signals and cues (e.g., shear stress and secreted molecules). Rho-GTPase activation, stress fiber formation, and actomyosin contraction are finely tuned through integrin adhesion and modulate junctional integrity (29). A recent study demonstrated that the integrin-associated focal adhesion tyrosine kinase (FAK) contributes to impairment of vascular barrier formation (30). VEGF-induced FAK activation was shown to lead to VE-cadherin/FAK interaction in association with β -catenin phosphorylation on tyrosine Y-142, resulting in dissociation of VE-cadherin and β -catenin, opening of junctions, and disruption of the endothelial barrier. Whereas adherens and tight junctions maintain the endothelial barrier, gap junctions connect adjacent endothelial cells as well as endothelial cells and mural cells (pericytes and smooth muscle cells) (31, 32). Water, ions, and small signaling molecules can be transferred via gap junctions, but gap junctions do not appear to contribute significantly to the establishment or maintenance of vascular barriers.

LEVELS OF VASCULAR PERMEABILITY

Vascular permeability is essential for homeostasis and varies in response to different physiological stimuli (temperature, exercise, etc.). There are important differences between the basal permeability and the changes that occur during pathology including whether the hyperpermeability is acute or chronic, the composition of extravasate, and the anatomic pathways that solutes follow in crossing vascular barriers. Under normal circumstances, water, lipophilic solutes (e.g., O₂ and CO₂), and small solutes are exchanged at the level of the capillary, driven by diffusion and plasma protein extravasation. Small molecules pass between endothelial cells, are transported via receptor-mediated transcytosis, or may cross through the fenestrae. Larger proteins, such as albumin, that are unable to pass between intact endothelial cells are instead transported via caveolae. The plasma membrane is heavily decorated with small, uncoated vesicles, which constitute this specialized microdomain (33). Most caveolae are connected to the luminal and abluminal plasma membranes—with few located on the lateral aspects of the cell. Based on studies using tracers, it was postulated that caveolae shuttled across capillary endothelium carrying plasma fluid and proteins (34). The role of caveolae, however, has been called into question by the findings that mice deficient for caveolin-1 (which therefore lack caveolae) are able to clear albumin more rapidly than their wild-type counterparts. Of note, caveolin-1-deficient mice exhibited 10–20% of the uncoated caveolae-sized vesicles present in wild-type mice although these vesicles did not stain for caveolin-1 (35).

In contrast to basal conditions, the protein content of the fluid that extravasates under circumstances of both acute and chronic vascular hyperpermeability approaches that of

plasma. The leak that accompanies acute hyperpermeability takes place at the level of the postcapillary venules (36). In contrast to the flattened endothelium of the capillary, postcapillary venules are lined by tall, cuboidal endothelium that utilizes VVOs for plasma extravasation (18, 37, 38). Although little is known about the mechanism of VVO function, it has been demonstrated using macromolecular tracers that exposure to permeability agents such as histamine or VEGF can induce the formation of interconnected VVO vesicles and vacuoles from the vascular lumen to the albumen, providing a transcellular pathway for plasma and plasma protein extravasation (18).

The leak that characterizes chronic hyperpermeability induces profound changes in venular structure and function. A category of new vessels termed “mother” vessels appears to be involved in pathological angiogenesis (39). Mother vessels are highly abnormal vascular structures that form in tumors and in other cases of abnormal neovascularization (39, 40). These vessels develop within hours after injection of tumor cells into mice, and are also seen in healing wounds and in tissues from nude mice that have been injected with an adenovirus expressing VEGF-A¹⁶⁴ (39, 40).

Structurally, mother vessels are dilated sinusoids that arise from preexisting normal venules by a process that involves basement membrane degradation, pericyte detachment, endothelial cell thinning, and a significant increase in lumen size. Two important components of the basement membrane, collagen IV and the laminin β 1 chain, have been reported to be degraded in the developing mother vessels both in tumors and after adenoviral-VEGF-A¹⁶⁴ injections (41). Of note, experimentally induced pericyte loss in the adult murine retinal microvasculature has been shown to be associated with microvascular permeability (Figure 2) and microaneurysms (42). In addition, formation of mother vessels involves reduction in several endogenous cysteine protease inhibitors and cystatin A, B, and C. Pericytes of venules that were remodeled into mother vessels have been shown to have increased cathepsin expression and activity (41).

REGULATORS OF VASCULAR PERMEABILITY

Vascular Endothelial Growth Factor

In the 1980s, vascular permeability factor (43), also known as VEGF (44), was identified and shown to be a key regulator of permeability (45). The VEGF family comprises a number of closely related ligands, including VEGF-A, B, C, D, and E and placental growth factor. Among these, VEGF-A (hereafter referred to as VEGF) is the best characterized. Various cell types, including arterial endothelial cells (46), smooth muscle cells, fibroblasts, a wide range of epithelial cells (47), parenchymal cells of a number of tissues and organs (e.g., liver and skeletal muscle) (47) and immune cells (macrophages, lymphocytes, neutrophils, eosinophils, and mast cells) produce and release VEGF, where it can act upon endothelial cells as well as a variety of nonendothelial cells (48). In addition, VEGF, which is encoded by a single gene, is differentially spliced into a number of isoforms—four common and three rare isoforms that vary from 121 to 207 amino acids in humans (49–52). Evidence from mice that have been engineered to produce single VEGF isoforms indicates that the isoforms serve distinct roles. It has been shown that hypoxia and other stress conditions induce the expression of VEGF by many cell types; moreover, VEGF is highly expressed in

tissues undergoing growth or remodeling—for example, during cancer, atherosclerosis, and remodeling of the female reproductive cycle.

Vascular permeability factor:

a glycoprotein that increases blood vessel permeability, endothelial cell growth, and angiogenesis, contributing to the increased neovascularization and vessel permeability associated with tumor vasculature

Members of the VEGF family signal through tyrosine kinase receptors (VEGFRs), in particular VEGFR1 (flt-1) (53), VEGFR2 (flk1 in the mouse, KDR in the human) (54), VEGFR3 (flt4) (55), and neuropilin (56). VEGF interacts with both VEGFR1 and -R2, but only VEGFR2 is directly involved in normal and pathological vascular permeability (57). Binding of VEGF to its VEGFR2 induces their dimerization and activation of an intracellular tyrosine kinase activity, recruitment of SH2 domain binding proteins, and subsequent downstream signaling through multiple common signaling pathways (55). VEGFR1 is reported to act as a negative regulator of VEGFR2 signaling by sequestering VEGF.

VEGF stimulation of endothelial cells in culture has been shown to stimulate virtually all known signaling pathways, with more than 50 different signaling molecules identified as being activated (Table 1). For instance, VEGF stimulates phospholipase C and A (58), protein kinase B (Akt) and PI3 kinase (59). VEGF is an endothelial mitogen, a powerful vascular permeability agent (60), and a potent vasodilator (61). It increases migration of endothelial cells, and has effects on the pericytes that surround endothelial cells (62) and on matrix metalloproteinases secretion by endothelial cells (63). Systemic VEGF administration results in hypotension via its effects on nitric oxide (64), whereas local secretion leads to vascular leak (65) and increased angiogenesis (66). Recently it has been shown that VEGF induces unequal responses depending on whether it acts on the luminal or abluminal side of neural microvascular endothelial cells; increasing VEGF levels within the lumen did not affect baseline permeability, whereas exposure to VEGF from the tissue side led to a rapid increase of permeability (67). In addition, cerebral and retinal vascular permeability was completely refractory to circulating VEGF, whereas the lung vasculature responded to circulating VEGF with enhanced permeability and p38 activation (67).

Vascular Endothelial Cadherin

VEGF activation of endothelial cells in culture results in phosphorylation and disassembly of VE-cadherin, one of the principal signaling and structural proteins associated with adherens junctions (115). VE-cadherin phosphorylation is initiated early (within 5 min) and peaks at 30 min after VEGF stimulation, strongly implicating it in the regulation of permeability. In support of this concept, there is considerable evidence demonstrating the regulation of adherens junctions during VEGF induction of vascular permeability. VEGF has also been shown to promote the phosphorylation of VE-cadherin and of its binding partners β -catenin, plakoglobin, and p120, in a Src-dependent mechanism (116). Consistent with this, VE-cadherin is not phosphorylated in Src-deficient mice (116). Furthermore, VE-cadherin

has been reported to associate with VEGFR2, an association that inhibits VE-cadherin phosphorylation and internalization (117). This interaction is speculated to potentiate the phosphorylation of VEGFR2 via adherens junction components by Src, thereby leading to impaired endothelial barrier integrity (118).

The association between VE-cadherin and VEGFR2 also contributes to VE-cadherin-induced contact inhibition of cell growth, an event that requires the β -catenin, but not the p120-catenin, binding domain of VE-cadherin (117). In addition, VEGF mediates VE-cadherin phosphorylation and internalization via the activation of Src, the guanine nucleotide exchange factor Vav2, the Rho-GTPase Rac, and p21-activated kinase (PAK)—a downstream effector molecule of Rac that has been implicated in cytoskeletal remodeling and cell motility (119). VE-cadherin is the target of a plethora of signaling events, which can provoke endothelial barrier disruption and vascular permeability increase (Table 2). Endothelial permeability is also reported to be regulated by changes in the expression of the components of adherens and tight junctions (120); mice lacking serum response factor, which is important for VE-cadherin expression (121), displayed reduced VE-cadherin expression.

Sphingosine-1-Phosphate

Sphingosine-1-phosphate (S1P), an important regulator of postcapillary venule permeability, acts through two receptors, S1P₁ and S1P₂, which are differentially expressed in various vascular endothelial beds. Detailed features of the S1P receptors involved in vascular permeability are shown in Table 3. S1P receptors mediate a range of vascular functions, such as vascular morphogenesis and maturation, cardiac function, vascular permeability, and tumor angiogenesis (150). It is well established that S1P, acting via the S1P₁ receptor, promotes endothelial cell barrier integrity. Intravenously delivered S1P attenuates vascular barrier dysfunction in murine and canine models of acute lung injury (150). Furthermore, FTY720, a high affinity agonist of S1P receptors, induces adherens junction assembly in the endothelial cell monolayer whereas oral FTY720 administration in mice potently blocks VEGF-induced dermal vascular permeability in vivo (151). More recently, bone-marrow derived progenitor cells from wild-type mice were shown to act through the S1P pathway to enhance endothelial barrier function as well as prevent increased vascular permeability and edema formation (152, 153).

Sphingosine-1-phosphate (S1P):

a major regulator of vascular and immune systems formed by the metabolism of sphingomyelin

In endothelial cells, the activation of the S1P₂ receptor results in disruption of adherens junctions and increased paracellular permeability, whereas administration of a S1P₂ receptor antagonist significantly inhibits H₂O₂-induced permeability in the rat lung perfused model (151). Induction of retinal hypoxia in mouse retinas that lack the S1P₂ receptor leads to significantly decreased inflammatory cell infiltration and enhanced revascularization of

the retina tissue, indicating that the S1P₂ receptor activates inflammatory pathways that facilitate vascular permeability and pathological angiogenesis (175).

Angiopoietin-1

Angiopoietin (Ang)-1, a ligand of the Tie-2 receptor, blocks vascular permeability; mice that overexpress Ang-1 are resistant to proinflammatory agents (176). In the mature vasculature, Ang-1 acts by inducing Rac1 activation and RhoA inhibition. p190 RhoGAP, a GTPase regulatory protein, is phosphorylated by Ang-1, requires Rac1 activation, and is necessary for RhoA inhibition (177). Thus, Ang-1 has been shown to protect against endotoxin-mediated vascular leak by regulating the endothelial cytoskeleton through coordinated and opposite effects on the Rho GTPases Rac1 and RhoA. At the cellular level this may translate to the regulation of intercellular gaps, as adenoviral delivery of Ang-1 has been demonstrated to attenuate leakage from bradykinin-induced pulmonary permeability by reducing the number and size of interendothelial gaps (178). Whereas Ang-1 reduces a VEGF-stimulated increase in blood-brain barrier permeability by the upregulation of ZO-2, a component of tight junctions (179), Ang-2, a naturally occurring antagonist of Ang-1, leads to microvascular destabilization both in an in vitro model (180) as well as in the blood-brain barrier in vivo (181).

Angiopoietin (Ang)-1:

a protein with important roles in vascular development and angiogenesis, encoded by the gene ANGPT1

Rho Family GTPases in Permeability

Members of the Rho family GTPases regulate endothelial barrier function; Rho family GTPases influence the actin cytoskeleton, directly modulating the integrity of endothelial cell-cell junctions. Rho A can lead to the disruption of barrier function through activation of Rac1 whereas activation of Cdc42 stabilizes cell-cell junctions. With expanding knowledge of Rho family GTPase regulation, new therapeutic opportunities to inhibit the proper permeability activity of RhoA and/or enhance the antipermeability of Rac1 and Cdc42 are possible. Thrombin increases Rho activation, whereas a delayed activation of Cdc42 contributes to the reassembly of interendothelial junctions and barrier integrity (182).

Rho family GTPases:

a small family of G proteins that have been shown to regulate many aspects of intracellular actin dynamics

Rac1 is important in the assembly and disruption of adherens and tight junctions. GTP-bound Rac1 and Cdc42 bind to and facilitate the autophosphorylation of PAK, which induces barrier breakdown, whereas blockade of PAK activity preserves barrier integrity (183). Downstream of PAK, activation of extracellular signal-regulated kinase is required for increased vascular permeability, and PAK-mediated extracellular signal-regulated kinase activation requires the formation of a complex containing PAK, PIX, and GIT1 (184). Thus,

blockade of the binding of PAK to PIX inhibits edema during acute lung injury (184). In addition, whereas PAK is activated in atherosclerosis-prone regions of arteries, inhibition of PAK phosphorylation reduces permeability during atherosclerosis in vivo (185). Of note, Rac, in particular, appears to be linked to VEGF-induced fenestration formation (186).

Transforming Growth Factor- β 1

Transforming growth factor (TGF)- β 1 is a multifunctional growth factor that is a well-established modulator of vascular cells (187). In vitro studies indicate that TGF- β 1 is activated upon contact between endothelial and mesenchymal cells (188) and that it mediates a variety of actions associated with vessel maturation including inhibition of endothelial cell proliferation and migration, induction of pericyte differentiation, and production of basement membrane (188–190). TGF- β signals through TGF β RII to recruit and phosphorylate TGF β RI, with subsequent downstream recruitment of smad transcription factors (191). Blockade of TGF- β signaling by systemic expression of endoglin (TGF β RIII) results in breakdown of the blood-retinal barrier, characterized by a decreased association between the tight junction proteins ZO-1 and occludin. Recently, it was reported that endoglin deficiency in vivo leads to an increase in endothelial cell permeability through constitutive activation of RhoA and destabilization of the endothelial barrier function. Destabilizing factor thrombospondin-1 and its receptor-like protein tyrosine phosphates are increased, whereas stabilizing factors VEGFR2 and VE-cadherin are decreased, suggesting that endoglin deficiency leads to endothelial cell hyperpermeability (192). The TGF β RI receptors ALK5 and ALK1 were shown to be involved in the regulation of permeability as signaling through ALK5-stabilized vascular endothelium, whereas signaling through ALK1 limited ALK5 signaling, leading to vascular destabilization (193, 194). These findings implicate TGF- β in maintaining the vascular barrier integrity and function of the normal retinal microvasculature.

Transforming growth factor (TGF)- β 1:

a multifunctional set of peptides that controls proliferation, differentiation, and other functions in many cell types

In primary breast tumors, TGF- β 1 activity is associated with an increased risk of lung metastasis (195). Indeed, Ang-related protein 4 (Ang-4), a target of TGF- β 1, disrupts endothelial cell-cell junctions, facilitating the extravasation of breast cancer cells (195). Moreover, TGF- β 1 induces the expression of VEGF in pericytes (196) and fibroblasts (197), whereas it inhibits Ang-1, an antipermeability factor, therefore exacerbating tumor-associated vascular leak (198).

ASSESSMENT OF BARRIER FUNCTION IN CULTURED ENDOTHELIAL CELLS

Because of the complexity of performing molecular or pharmacological manipulations to study barrier function in vivo, cultured endothelial cells are often used to model the microvascular endothelium, and most involve confluent endothelial cells cultured on porous

membrane filters in transwell chambers. A number of in vitro assays have been developed to measure molecular flux (Table 4). The transwell assay is widely used to determine the transendothelial permeability of solutes (e.g., albumin, dextrans, and sucrose) across endothelial monolayers (199). These systems have a number of advantages including that they are easy to perform and avoid the complexities of living animals (described below). It goes without saying that such assays do not come without limitations. Generally, monolayers of cultured endothelial cells are not able to form barriers that are as tight as their in vivo counterparts. This difference is likely due to a number of variables including the fact that the cultures lack normal basement membrane (200), contributions from other cell types (e.g., pericytes or smooth muscle cells) are missing (18), and cytoplasmic vesicles and vacuoles, structures that are important for macromolecular extravasation, are not observed at the levels seen in vivo (201). In addition, the conditions of tissue culture are distinct from that experienced by the cells in vivo and involve the presence of serum and growth factors and the growth of cells on a plastic substratum that lead to changes in gene expression that differ from in vivo. As such, the kinetics of permeability in response to agents such as VEGF differs significantly from that of in vivo settings. In vivo leakage in response to a single exposure of VEGF begins rapidly and is fully complete within 20–30 min. In contrast, changes in permeability often develop more slowly in cultured endothelium and may continue over a period of hours.

Endothelial cell membranes are composed of lipophilic molecules (e.g., phospholipids and cholesterol) that restrict the passage of aqueous solutions and function as electrical insulators to maintain the physiological endothelial membrane electrical potential. Thus, the fluid-filled pores at cell-cell junctions can also function as electrical conductance pathways across the endothelium. In theory, an increase in the number of open pores can be correlated to a decrease in electrical resistance across the microvascular endothelium. The measurement of electrical resistance across microvessel walls [i.e., transendothelial electrical resistance (TER)] is relatively simple. Using transwells, TER is measured directly across the cell monolayer using an electrical resistance meter with probes placed into both the luminal and abluminal compartments. Barrier function is calculated based on impedance to the flow of small inorganic ions across the microvessel wall. An advantage of this model is that it also allows the examination of interactions between different cell types that form protective barriers and as such a coculture model is often used (Figure 3).

Transendothelial electrical resistance (TER):

a measurement that provides a quick and easy evaluation of blood-brain barrier integrity

Other quantitative methods for determining the TER include the use of fluorescently labeled dextran to characterize changes in endothelial cell permeability to higher molecular weight molecules. In this case, transfer is measured by relative fluorescence as well as by the use of an electrical cell-substrate impedance sensor (203), a device that quantifies cell density, migration, and electrical impedance.

Electrical cell-substrate impedance sensor:

a noninvasive biophysical approach to monitor living animal cells in vitro

Thus, although in vitro systems to study vessel permeability have been well studied, these models have limited resemblance to real physiological structures and functions. In many cases, however, cultured endothelial cells are the only available model for testing mechanistic hypotheses related to cell signaling in control of microvascular barrier function, as it is often not technically possible to perform these kinds of experiments in vivo.

EVALUATION OF VASCULAR LEAK IN MICE

Miles Assay

The Miles assay has been widely used to assess and to quantify vascular permeability in vivo (204). Although this assay was originally developed using the skin of guinea pigs, it has since been adapted to a number of different mouse organs such as the liver and ear, to name a few (205). In brief, this assay uses an intravenously injected tracer, Evans blue, a dye that binds noncovalently to albumin, to measure dye accumulation at the test site (Figure 4). Permeability is defined by the amount of albumin-dye complex present at an indicated time point after Evans blue injection. Visual discoloration or bluing at the site of injection reflects leakage from the vascular compartment; quantitative measurements can also be made by extracting the dye from tissues and measuring it spectrophotometrically (206). Although the Miles assay is widely employed, there are some limitations to this assay. For one, it is not suitable for the assessment of vascular permeability in noninflamed tissue because the albumin (which is tagged by the tracer) does not traverse the vascular barrier in significant quantities in the absence of inflammation. Another major limitation is that the assay measures net accumulation of dye-albumin complex over a period of time, so that the return of extravasated molecules to the circulation, by way of either capillaries or lymphatics, is not considered. Regardless of these limitations, the Miles assay provides useful information regarding permeability of the vascular compartment of a specific tissue.

First-Order Model of Albumin Kinetics Using a Dual Isotope Approach

In instances where the vasculature undergoes pathological changes it is possible to measure both the content of a macromolecular tracer and the dye that has extravasated, allowing investigation of both structural and functional changes of the vasculature. This is achieved using a dual tracer method (e.g., combining ^{131}I - and ^{125}I -albumin or Evans blue dye and ^{125}I -albumin) (205), where the first tracer is used to measure total albumin accumulation over a course of 30 min, and the second tracer, injected 5 min before tissue harvest, measures albumin that is present within the vasculature. The difference between these measurements allows for quantitation of intravascular plasma volume and the volume of leaked plasma.

Ultrastructural Visualization of Permeability

Structural alterations of the endothelium with increased permeability can be visualized by transmission electron microscopy (TEM). Initially, TEM was utilized to visualize the

structural aspects of the vascular endothelium (200) but was later used to study vascular leakage from VEGF-stimulated and tumor-associated blood vessels (37). More recently, TEM was employed to show that heart or brain capillaries exposed to systemic VEGF respond with a series of events including development of gaps between adjacent endothelial cells and attraction of platelets to exposed basement membrane (118). Of note, assessment at the ultrastructural level can provide insights into the mechanism by which an agent promotes or blocks vascular leak.

Evaluation of Vascular Leak Using Light Microscopy

Taking advantage of the unique binding properties of specific lectins, labeling with Griffonia simplicifolia lectins is used to measure permeability *in vivo*. In this method, vascular leak is measured by light microscopy after perfusion with lectins; peroxidase is used to visualize the binding of lectin to specific locations during the permeability response in tissue sections of whole-mount preparations (207). In addition to lectins, extravasation of labeled tracers such as fluorescently labeled dextrans can also be employed to detect both vessel perfusion and leak. Dextrans of different sizes can be used to evaluate the contribution of different pathways to the leak response (i.e., fenestrations, caveolae, VVOs, endothelial gaps). Use of different sized microspheres or different molecular weight fluorescently-labeled dextran (4–70 kDa) can be applied to better assess the extent of endothelial leak. Depending on the molecular size, these tracers can be used to test both solute and ion permeability (low molecular weight dextrans) and protein permeability (high molecular weight dextrans). Movement of dextrans out of the plasma through the fenestrae and channels is relatively rapid (3–7 min). This can create transient concentration gradients in the pericapillary space centered on the fenestrated sectors of the capillary walls. Recently, passage of dextran larger than 70 kDa was shown to be retained in skin blood vessels under homeostatic conditions, whereas under inflammatory conditions, the hyperpermeability was selectively induced in the postcapillary venules and dextran as large as 2,000 kDa leaked from the blood *in vivo* (208). Together, use of electron and light microscopic techniques offer valuable insights into the structural determinants of leakage within pathological samples.

IMPLICATIONS OF VASCULAR PERMEABILITY DURING PATHOLOGY

Under physiological conditions, microvascular permeability is tightly regulated. However, during a variety of pathologies, the disruption of the vascular barrier results in the release of potent permeability agents, as well as agents that recruit platelets and leukocytes to the affected site. Aberrant and uncontrolled vascular hyperpermeability can participate in the progression of many pathological states, such as chronic inflammatory diseases, diabetes, tumor angiogenesis, and retinal diseases. This section highlights current knowledge regarding how vascular leak impacts the progression of various diseases and examines the promise of new clinical therapies aimed at controlling excess permeability. Other review articles provide further details on angiogenic diseases (209), antiangiogenesis strategies (210), and preclinical and clinical advances of vessel normalization (6). The reader is referred to other excellent reviews to learn more about new therapeutic approaches that are currently being used for cancer and other angiogenic disorders (211–213).

Cancer and Metastasis

Hypoxia is a principal driver of VEGF expression and is considered to be one of the primary inducers of increased vascular supply to tumors. The VEGF produced by tumor cells stimulates not only angiogenesis but also increased vascular permeability with resulting edema. Inhibition of VEGF has been shown to reduce the fluid accumulation in and around tumors, reducing morbidity or metastasis in experimental models (214–216). Since its approval by the US Food and Drug Administration (FDA) in 2006, the anti-VEGF agent bevacizumab (Avastin[®]) has been used in combination with drugs, chemotherapeutic agents, or other antibodies in an attempt to block tumor vascularization. In spite of initial hopes that antiangiogenesis would have high efficacy in tumor suppression, results in human cancer lack success. Bevacizumab, when administered with standard chemotherapy, results in a modest increase in survival (e.g., 2–5 months) in certain cancers (colorectal and lung) but not others (pancreatic and breast) (217, 218). Similarly, two broad-spectrum tyrosine kinase inhibitors that also target VEGF receptors (e.g., sunitinib and sorafenib) have also had limited success (219, 220). Although these therapies often prolong survival by a matter of months, they generally do not markedly extend life. A number of explanations have been offered for the modest effectiveness of anti-VEGF/VEGFR drugs in treating human cancers, whereas more potent effects are observed in tumor-bearing mice. One possibility is the need for better dosing strategies, as the fragility of cancer patients means that they cannot withstand the toxicities associated with the high dose therapy that can be used in tumor-bearing mice. Another reason could be that anti-VEGF/VEGFR therapy does not kill all tumor cells and the residual tumor cells that remain behind are rendered hypoxic and become stimulated, making increased amounts of VEGF-A that may overwhelm the anti-VEGF/VEGFR therapy and thus protect the cells from anti-VEGF drugs (221). Other explanations include vascular normalization, a period of time following anti-VEGF/VEGFR therapy in which tumor vessels normalize structurally and lose their hyperpermeability.

Despite the lack of success of anti-VEGF/VEGFR therapy for most cancers, the utility of VEGF antibodies in the treatment of ovarian carcinoma was initially explored in animal models where VEGF blockade was shown to inhibit the formation of ascites and slow tumor growth (222). The role of bevacizumab in the management of ovarian cancer has been examined in two randomized phase II trials: Gynecologic Oncology Group and Genentech AVF 2949; although there was a statistically significant improvement in progression-free survival, no improvement in overall survival was reported (223). In 2014, the FDA approved bevacizumab as a treatment for ovarian cancer. Of note, the FDA has also approved the anti-VEGF bevacizumab to treat glioblastoma, advanced nonsquamous, and non-small-cell lung cancer.

Although VEGF has been the most widely exploited target for antiangiogenic therapy, increased attention has been directed toward the Ang–Tie2 pathway, in particular by inhibiting Ang-1 and/or Ang-2 with monoclonal antibodies (224). Currently, there are a number of agents targeting the Ang/Tie2 axis under investigation in clinical trials. One example is AKB-9778, a selective inhibitor of vascular endothelial protein tyrosine phosphatase (VE-PTP), which promote maturation of tumor vessels by blunt tumor growth via Tie2 activation (225). Inhibition of Ang-2 has been shown to mitigate vascular

leak syndrome in patients receiving interleukin (IL)-2 treatment. In addition, blocking endogenous hyaluronan with a hyaluronan-specific binding peptide has been reported as a novel means to prevent IL-2-induced vascular leak syndrome. The region of IL-2 that mediates the induction of vascular leak syndrome has been identified as a shared three-amino acid consensus motif, (x)D(y), believed to be responsible (226). Currently, an immunotoxin engineered to eliminate vascular leak syndrome is being used in preclinical models in combination with IL-2 to treat cancer (226).

Ischemic Diseases

Changes in blood-brain barrier permeability are a characteristic of ischemic stroke. Vascular permeability has been reported to precede spontaneous intracerebral hemorrhage in stroke-prone spontaneously hypertensive rats (227). The edema associated with stroke is a primary cause of neuronal damage and also impedes recovery. Thus, inhibitors that reduce initial edema in the brain and heart have been shown in experimental mouse models to limit infarct size, increase function, and prolong survival. From a more mechanistic perspective, VEGF is produced following myocardial infarction or stroke in response to resultant hypoxia. Mice lacking the Src family kinases Src or YES proto-oncogene 1 do not exhibit vascular permeability in response to VEGF (228) and thus have reduced infarct size and edema following ischemic stroke. Similar protection is observed with use of VEGF antagonists or pharmacological Src family kinase inhibition (118).

Pulmonary Diseases

Pulmonary conditions that involve endothelial barrier hyperpermeability, including asthma, acute lung injury, and ventilator-induced lung injury, are associated with increased vascular permeability that, in the latter settings can result in pulmonary edema. It has been proposed that the increased vascular permeability in ventilator-induced lung injury is a result of an active endothelial response to mechanical strain, which induces signaling pathways that lead to vascular permeability. It has also been shown that lung injury activates different PI3K-mediated pathways that oppose each other and that the balance between these pathways determines the net effect on vascular permeability (229). Treatment with inhibitors of PI3K or Src family kinases can also reduce edema in the ventilated mouse lung, and activation of the PI3K-Akt-GSK3 β pathway tends to limit the permeability response by preserving expression of β -catenin (229).

Ocular Diseases

Hypoxia has been demonstrated to induce production of VEGF by retinal endothelial cells, retinal pericytes, Muller cells, retinal pigment epithelial cells, and ganglion cells (230–235). Furthermore, experimental induction of retinal ischemia in both mouse and primate eyes has demonstrated retinal (232) and iris (236) neovascularization, respectively, that are associated with elevated levels of VEGF and that can be blocked by VEGF neutralizing antisera (237). The majority of severe visual loss in the United States results from complications associated with retinal neovascularization in patients with ischemic ocular diseases such as diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity. Diabetic retinopathy, for example, occurs when capillary loss from the retina leads to localized areas of retinal nonperfusion. The resultant hypoxia leads to the production of VEGF by cells

in the ischemic retinal tissues, which leads to edema, leaky vessels and neovascularization causing disrupted vision. Anti-VEGF therapies are being widely used in the treatment of diabetic macular edema where it has been shown to be highly effective. VEGF neutralization is similarly efficacious as a therapy for central retinal vein occlusions as well as branch vein occlusions. However, the effect of anti-VEGF treatment for the management of retinopathy of prematurity is controversial because of the high level of active angiogenesis that is taking place in many, if not all, tissues and organs of these premature infants (238).

In the wet (or exudative) form of AMD, increased VEGF expression leads to proliferation of abnormal and highly permeable vessels from the choriocapillaris into the subretinal space. Whereas retinal blood vessels are part of the blood-brain barrier and have extremely low hydraulic conductivity (239), the choroidal vasculature has a very high permeability due to the presence of fenestrations in the endothelial cells (239). When newly forming and leaking choroidal vessels invade into the retinal space in the region of the macula, the fluid disrupts the retinal architecture, resulting in disruption of central vision. Intraocular injection of either bevacizumab, the variable fragment of a similar VEGF antibody (termed ranibizumab), or a VEGF-trap known as aflibercept, can initially reverse visual loss and restore vision (240) in a subset of patients.

Studies in rodents have begun to elucidate the function of VEGF in normal adult tissues. Systemic inhibition of VEGF using adenoviral expression of sFlt-1 leads to nonperfusion of vessels in the choroid plexus (47), death of photoreceptors that were shown to express VEGFR2 (241), and degeneration of the retinal pigment epithelium that was similarly demonstrated to express VEGFR2 (242). In addition, the choriocapillaris, as well as all of the other fenestrated vascular beds of the body, has been shown to be uniquely vulnerable to VEGF inhibition (243). Decreased fenestrations, vessel occlusion, and vessel regression were observed after intravitreal injection of anti-VEGF (bevacizumab) in primate eyes (244). Systemic inhibition of VEGF using either systemic ad-sFlt (47, 241, 245) or pharmacological inhibition (246) led to the loss of fenestration and atrophy of the vasculatures of the choroid plexus, choriocapillaris, ciliary body, pancreatic islets, thyroid, adrenal cortex, pituitary, small-intestinal villi, and adipose tissue (243).

The involvement of VEGF in proliferative AMD has led to the development of a number of VEGF blocking therapies. Clinical trials have shown that pegaptanib, an aptamer that blocks VEGF function and was approved for clinical use as Macugen[®], slows visual loss in the majority of patients with AMD, with some patients showing visual improvement (247). Ranibizumab, a humanized antibody fragment directed against VEGF marketed as Lucentis[®], was approved for the treatment of neovascular AMD (248) and has been shown to not only stabilize disease progression but also to frequently cause the partial restoration of vision. It is widely believed that the reversal of vision loss is due to the resolution of macular edema, indicating that these therapies are acting primarily as antipermeability agents.

CONCLUSION

Defective endothelial barrier function is a common feature of many disorders. Understanding the molecular basis of vascular permeability has led to new therapeutic

strategies to prevent vascular leakage, which have proven to be successful in a number of preclinical models. The identification of new strategies for reducing permeability and application of existing strategies to an expanding array of abnormal pathologies are the focus of much research. An improved understanding of the molecular and cellular pathways involved in the regulation of vascular permeability will be critical to developing more targeted, efficient, and safe therapies for treating a wide range of human diseases.

This review has provided a greater understanding of how vascular leak impacts the progression of various diseases. Despite significant progress, the field still lacks a comprehensive picture of the mechanisms by which different agonists induce either paracellular or transcellular permeability. Also, signaling pathways and proteins by which different agonists modulate vascular permeability continue to be a complex subject in need of further development. Fully understanding the function of VE-cadherin regulation, for example, would make it possible to design specific agents that can increase or decrease vascular permeability. Further work, therefore, is required to address these key topics in order to design new strategies for reducing permeability. The most important challenge for the future of this field will be to develop suitable treatments to regulate permeability, which may include more sensitive imaging tools to prevent vascular leak or better antipermeability agents to treat different pathophysiological conditions.

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Figure 1.

Transcellular and paracellular pathways in endothelial cells. The passage of macromolecules, fluids, and cells through the endothelial barrier can occur through transcellular (vesiculo-vacuolar organelles) or paracellular (tight and adherens junctions) pathways. Abbreviations: JAM, junctional adhesion molecule; VVO, vesiculo-vacuolar organelle; ZO, zona occludens.

Figure 2.

Pericyte loss in the retinal microvasculature is associated with microvascular permeability. iDTR;M-Cre mice were generated by breeding Cre-inducible DT receptor–transgenic mice [C57BL/6-Gt(ROSA)26Sor^{tm1(HBEGF)Awai/J} (annotated as iDTR)] with mice expressing tamoxifen-inducible Cre under the control of the SMMHC promoter (annotated as M-Cre). DT-treated iDTR;M-Cre mice were perfused with fluorescein dextran (2×10^6 mW) before retinal imaging by fluorescence microscopy. Arrows indicate sites of increased microvascular permeability in the retinal microvasculature of mice with inducible mural cell loss. Scale bar: 250 μ m. Abbreviations: DT, diphtheria toxin; SMMHC, smooth muscle myosin heavy chain. Unpublished photo by C. Valdez, J. Arboleda, and P.A. D'Amore.

Figure 3.

Astrocyte-endothelial cell cocultures and time course of TER. (*a-i*) Brain astrocytes were isolated from rat pups at postnatal day 1, plated onto tissue culture flasks, and allowed to grow for 7–10 days. Cells were cultured on tissue culture plastic, in Matrigel or on Transwell membranes for morphological comparison. (*a,b*) Astrocytes immunolabeled with GFAP were cultured (*a*) for 24 h alone or (*b*) in coculture with rat microvascular ECs on tissue culture plastic. Arrows indicate ECs next to astrocytes. Astrocytes and bovine microvascular ECs were grown alone and in coculture in a three-dimensional Matrigel assay for 24 h. (*c*) Astrocytes were labeled green and (*d*) ECs were labeled red to distinguish the cell populations. (*e,f*) Astrocytes and ECs were grown in Matrigel coculture. (*g-i*) Astrocytes and bovine microvascular ECs were grown on opposite sides of a Transwell membrane and identified with GFAP labeling (*red*) or simplicifolia lectin (*green*), respectively, to distinguish the cell types. The arrows indicate areas of astrocyte contact with ECs, and asterisks indicate areas shown in higher magnification. (*j*) ECs were plated onto 12 well Transwell inserts alone (*green triangles*), cocultured with astrocytes on the underside of the well (*orange circles*), or cocultured with BAE on the underside of the well (*blue squares*). TER was measured daily with a Millipore electrical resistance unit. TER from an empty Transwell was used as background and was subtracted from EC readings. The average of triplicate measures from each well was used to calculate TER/cm². Readings were measured from triplicate wells. Scale bar: 20 μ m in panels *a-e* and *g-i* and 40 μ m in panel *f*. Abbreviations: BAE, bovine aortic endothelium; EC, endothelial cell; GFAP, glial fibrillary acidic protein; TER, transendothelial electrical resistance. Figure adapted from Reference 202 with permission from Elsevier Limited.

Figure 4. Miles permeability assay. Different concentrations of VEGF-A were injected subcutaneously into mice, followed immediately by an intravenous injection of Evans blue dye. HBSS was injected as a control. Abbreviations: HBSS, Hanks' buffered saline solution; VEGF-A, vascular endothelial growth factor-A. Image courtesy of Janice Nagy and Harold Dvorak (Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115).

Table 1

VEGF-induced cell signaling and related biological effects in endothelial cells

Cell type(s)	Biological effect(s)	Signaling response(s)	Reference(s)
Human umbilical vein ECs	Cell survival; inhibition of apoptosis	KDR-mediated PI3K and Akt activation	59
Human umbilical vein ECs	Cell survival; gene expression	Upregulation of Bcl-2	59
Human umbilical vein ECs	Cell survival	Upregulation of XIAP and survivin	68
Human umbilical vein ECs Human dermal microvascular ECs	Cell migration	Akt-dependent eNOS phosphorylation PLD activation Rac1 activity Activation of Rac1 and Rac GEF; Vav2 association with G15ARac1	69–72
Human umbilical vein ECs	Cell migration; focal adhesion formation	ERK1/2 activation; tyrosine phosphorylation of PLC- γ and FAK α 3 integrin tyrosine phosphorylation via c-Src Integrin signaling via c-Src and FAK in CD63-ablated cells	73–75
Human umbilical vein ECs	Cell directional migration; vascular sprouting	Phosphorylation of PLC β 3 is dependent on the VEGF-VEGFR2 axis	76
Human umbilical vein ECs	Cell-cell contact; cell migration	Actin-binding protein IQGAP1	77
Human umbilical vein ECs	Cell migration and permeability	Phosphorylation of VE-cadherin, β -catenin, plakoglobin, p120, and PECAM-1	228
Human umbilical vein ECs	Cell migration; actin filament reorganization	Recruitment of vinculin to focal adhesions; ERK and p38 stimulation; Hsp 90–dependent FAK phosphorylation TSAd forms complex with Src Activation of SAPK2/p38 MAPK Generation of PIP3 activates Rac	78–82
Human umbilical vein ECs Porcine aortic ECs	Cell migration and proliferation	NO-mediated depression of PKC δ activity VEGFR2-pY1175/1173 is required for PI3 kinase activation Gq/11 and G β γ proteins are required for VEGF-stimulated intracellular Ca ²⁺ mobilization	83–85
Human umbilical vein ECs Bovine aortic ECs	Cell proliferation	PKC α and PKC ζ translocation and specific inhibition using antisense β 3 integrin and KDR-mediated PI3 kinase stimulation Raf-1 and PKG activation Ca ²⁺ and PI3K-mediated cGMP increase and eNOS upregulation PTP1B negatively regulates VEGFR2 autophosphorylation, inhibiting Erk1/2 phosphorylation Activation of PKD modulates Erk1/2 pathway	86–91
Bovine pulmonary artery ECs	Cell proliferation and permeability	Loss of β -catenin staining from adherens junctions; PLC- γ , paxillin, and β -catenin phosphorylation	92
Bovine retinal ECs Bovine microvascular ECs Porcine brain microvascular ECs Porcine isolated coronary venules	Cell permeability	Activation of ERK and cPLA2 NO and PGI2 production cGMP production PLC- γ phosphorylation; PKC-mediated eNOS phosphorylation	93–96
Human dermal microvascular ECs	Angiogenesis	Increased expression of a 1 β 1 and a 2 β 1 integrins	97
Human umbilical vein ECs Bovine aortic ECs	DNA synthesis	Phosphorylation of PLC- γ , FAK, AKT, and Erk; PKC ϵ translocation ERK activation ERK-mediated JNK activation; cyclin D1 synthesis; Cdk4 activation PKC α and PKC β translocation; PLC- γ phosphorylation; PI3K	98–101

Cell type(s)	Biological effect(s)	Signaling response(s)	Reference(s)
		activation NOS ser1177 phosphorylation	
Sinusoidal ECs Porcine aortic ECs	DNA synthesis; PKC- dependent ERK activation	Activation of Raf-1; Mek, ERK, and PKC β translocation Shc-Grb2 complex formation	102–104
Human umbilical vein ECs	NO production; gene expression	Upregulation of eNOS	88, 105
Postcapillary endothelial cells	NO-mediated ERK activation	NO production; ERK activation	106
Human umbilical vein ECs	Ca ²⁺ -independent NO production	Occludin and ZO-1 tyrosine phosphorylation	107, 108
Bovine aortic ECs	NO production; PGI production	Association of Src with KDR; PLC- γ activation; intracellular Ca ²⁺ mobilization	109
Human umbilical vein ECs	Tissue factor production	Flt1 activation	110
Bovine retinal ECs Bovine adrenal cortex ECs	Gene expression	ERK and PKC-mediated upregulation of Ang-2 PKC-mediated eNOS upregulation	111, 112
Porcine aortic ECs	VEGFR2-specific signaling and proliferation	Grb2 and Nck association with KDR; SHP1 and SHP2 association with KDR	104
Human umbilical vein ECs Porcine aortic ECs	VEGFR2-specific signaling	Ca ²⁺ mobilization NO and cGMP production	113, 114
Bovine retinal ECs	Intracellular trafficking	KDR, caveolin-1, and eNOS translocation to the nucleus	201

Abbreviations: EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; FAK, focal adhesion tyrosine kinase; PECAM-1, platelet-endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor.

Table 2

Breakdown of VE-cadherin junctions and the endothelial barrier

Route(s) of VE-cadherin disassembly and vascular permeability	Cellular function(s)	Reference(s)
VE-cadherin phosphorylation	Putative phospho-tyrosine sites of VE-cadherin are implicated in barrier integrity VE-cadherin serine phosphorylation modulates adherens junction assembly VE-cadherin/catenin complexes hamper vascular permeability and leukocyte extravasation Nonreceptor tyrosine kinase Src leads to phosphorylation of VE-cadherin and catenins, and to adherens junction disassembly Src-directed VE-cadherin phosphorylation is insufficient to drive endothelial barrier opening FAK mediates adherens junction disorganization and permeability	30, 119, 122–131
VE-cadherin phosphorylation	Activation of small GTPases, including RhoA, Rac, and cdc42, and myosin light chain phosphorylation depend on cellular context and extracellular cues Rac mediates VEGF-induced permeability upon VE-cadherin transactivation	132, 133
VE-cadherin and mechanical forces	Regulation of cell-cell adhesion and endothelial barrier function Endosomal trafficking through clathrin-coated vesicles Migration and permeability p120-catenin modulates VE-cadherin endocytosis PI3K signaling is involved in VE-cadherin internalization and loss of vascular barrier Antipermeability agents operate through the blockade of VE-cadherin internalization	119, 120, 123, 128, 134–142
VE-cadherin availability	KLF4 depletion leads to VE-cadherin reduction and loss of adherens junctions and barrier integrity Wnt3a/ β -catenin signaling controls claudin-3 expression and barrier maturation Loss of catenin p120 leads to VE-cadherin downregulation, microvasculature disorganization, and loss of vascular integrity in mice miR27a, miR101, miR125b, and miR142a modulate VE-cadherin expression, angiogenesis, and vascular permeability	143–149

Abbreviations: FAK, focal adhesion tyrosine kinase; VE-cadherin, vascular endothelial cadherin.

Table 3

Sphingosine-1-phosphate receptor subtypes involved in vascular permeability

S1P subtype	Tissue expression	Function(s)	Reference(s)
S1P ₁	Brain and heart Spleen and liver Lung, thymus, kidney, and skeletal muscle Lymphoid	Astrocyte migration Blockade of egress and chemotaxis in B cells Increase in β -AR-positive inotrophy induces calcium overload in cardiomyocytes Endothelial cell: early vascular development; adherens junction assembly; APC-mediated increased barrier integrity; Rho-dependent activation of integrins; activation of matrix-degrading proteinases; barrier protection Neural stem cell migration Pericyte: early vascular development adhesion protein and barrier integrity Blockade of egress and chemotaxis decreased late-stage maturation in T cells Inhibition of Rac activation; recruitment of VSMCs	134, 154–167
S1P ₂	Brain and heart Spleen and liver Lung, thymus, kidney, and skeletal muscle	Cardiomyocyte survival Apoptotic cell extrusion of epithelial cells Endothelial cell: pathological angiogenesis adherens junction disruption Proliferation and matrix remodeling in hepatocyte Mast cell degranulation Reduced PDGF-induced migration of VSMCs	168–175

Abbreviations: S1P, sphingosine-1-phosphate; VSMC, vascular smooth muscle cell.

Table 4

Methods to evaluate vascular permeability

	Model	Description	Advantage(s)	Disadvantage(s)
In vitro	Transwell assay	Measures the accumulation of tracer in lower chamber	Visualizes cells using microscopy and immunohistochemistry	Relies on absolute confluence of monolayer
	Transendothelial resistance	Measures changes in resistance or impedance across confluent monolayer	Measures rapid and reversible responses	Represents cell-cell and cell-matrix interactions
	Electrical cell-substrate impedance sensor	Analyzes the impedance spectrum of adherent cells using electrodes	Measures impedance and can differentiate between junctional impedance and impedance caused by cell-substrate interactions	Does not provide direct information on the molecular level
	Microcarrier beads	Assesses tracers that pass through cell monolayers into a bead matrix	Measures very rapid changes	May have heterogeneous endothelial cell confluency among microcarrier beads
In vivo	Miles assay	Extravasates tracer from blood vessels in the skin via local injection of a permeability-inducing agent	Quantifies labeled tracers by extraction	May vary depending on vessel morphology in skin
	First-order model of albumin kinetics using a dual isotope approach	Measures tracers to estimate the permeability-surface area for albumin	Measures rate of albumin leakage into tissue Simple to implement in a small animal model	Does not account for kinetic details such as backdiffusion or lymphatic drainage
	Evaluation of vascular leak	Uses microscopy to visualize movement of injected tracer Extravasates labeled tracers such as dextrans and <i>Griffonia simplicifolia</i> lectins to detect vessel perfusion and leak	Light, fluorescence, or electron microscopy can be used to evaluate vascular leak Dextrans of different sizes can be used to evaluate the contribution of different pathways to the leak response	Requires tissue manipulation Individual vessels may not be representative of entire vascular beds
	Intravital microscopy of vascular beds	Extravasates labeled tracers to measure thin tissue preparations	Identifies sites of leak and can quantify extent of extravasated tracer	Requires tissue manipulation and is difficult to use for long-term studies