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Lena Claesson-Welsh

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REVIEW ARTICLE

Vascular permeability—the essentials

LENA CLAESSION-WELSH

Uppsala University, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Uppsala, Sweden



Winner of the Rudbeck Award, 2014, at the Medical Faculty of Uppsala University for her distinguished research on the formation and function of blood vessels under normal and pathological conditions.

Abstract

The vasculature, composed of vessels of different morphology and function, distributes blood to all tissues and maintains physiological tissue homeostasis. In pathologies, the vasculature is often affected by, and engaged in, the disease process. This may result in excessive formation of new, unstable, and hyperpermeable vessels with poor blood flow, which further promotes hypoxia and disease propagation. Chronic vessel permeability may also facilitate metastatic spread of cancer. Thus, there is a strong incentive to learn more about an important aspect of vessel biology in health and disease: the regulation of vessel permeability. The current review aims to summarize current insights into different mechanisms of vascular permeability, its regulatory factors, and the consequences for disease.

Key words: *Edema, histamine, junctions, pore, vascular permeability, VEGF*

Introduction

The main function of the vasculature is to serve as a blood conduit to ensure efficient oxygenation of tissues, followed by the return of the deoxygenated blood to the lungs. The vasculature is also pivotal for a range of other homeostatic functions relating to the circulation such as hemostasis, lipid transport, and immune surveillance (1). Endothelial cells are the main constituents of blood vessels. They rest on a basement membrane with their basolateral side and face the blood with their apical/luminal side.

In the healthy individual, the vasculature is stable, and endothelial cell survival is continuously

maintained (2). 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. In fact, the growth of all new tissues, whether healthy or not, is accompanied by blood vessel formation. A main underlying mechanism is the relative hypoxia in the growing tissue (3). During embryogenesis, vessels form *de novo* in a process denoted vasculogenesis, while angiogenesis implies vessel formation from the pre-existing vasculature.

Endothelial cells in different vessels and in different organs have distinct functions and morphologies (4). In certain organs, such as the brain and in endocrine

organs, endothelial cells present certain morphological features that reflect the need for communication between the organs and the circulation. In the brain, the vasculature forms a particularly strong barrier, the blood–brain barrier (BBB) (5), to protect the brain parenchyma from detrimental edema. In hormone-producing organs, such as the endocrine pancreas, endothelial cells display specialized fenestrae on their surface. These are diaphragm-covered ‘holes’ in the plasma membrane, which allow extremely rapid exocytosis of hormones (6).

In most organs, the endothelial cells form a dynamic barrier between the blood and the tissue. In resting conditions, the vasculature continuously leaks solute and small molecules 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.

This review will discuss the current knowledge of how different types of vascular permeability are regulated, how regulation is lost in diseases, and, finally, how insights into regulatory mechanisms can be exploited therapeutically.

Mechanisms in permeability

Traditionally, the term ‘vascular permeability’ implies the basal vascular sieving of solute and small molecules, which occurs in an unstimulated setting. Molecules smaller than 40 kDa may extravasate spontaneously (7), whereas larger molecules require the active disruption of the vascular barrier in order to extravasate to the surrounding tissue. Such induced leakage takes place preferentially in post-capillary venules (8,9), but capillaries and larger venules may also leak (10).

The mechanism underlying vascular leak may be different in different organs and depend on the specialized vasculature. However, two main models have been proposed. One depends on formation of transendothelial channels from vesicles or vacuoles, the vesiculo-vacuolar organelle (VVO), and the other involves endothelial junctions that can be transiently dissolved and allow extravasation. The actin cytoskeleton may have a critical role in gap formation. Moreover, the specialized junction in the brain vasculature, instrumental in the BBB, and the features of fenestrated endothelium will be described. The involvement of these different mechanisms may depend on the vessel type, the organ, the kinetics of the transport, and the nature of what is transported

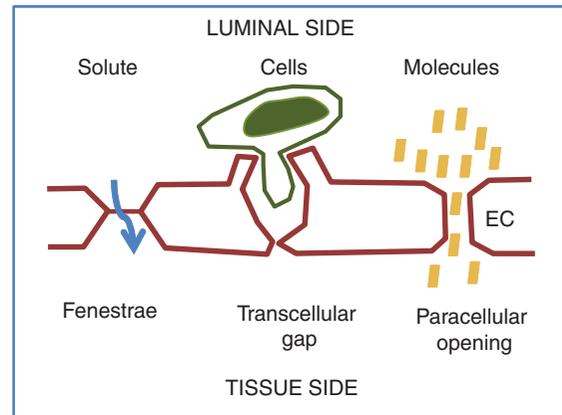


Figure 1. Different mechanisms for extravasation of solute, cells, and molecules. Specialized capillaries in endocrine organs have pores, fenestrae, in the plasma membrane. Fenestrae allow rapid exchange of solute and molecules such as hormones. Transcellular gaps provide a route for inflammatory cells, which, however, also may exit through paracellular junctions. Disintegration of junctions allows extravasation of molecules.

across the vascular wall—solute, molecules, or cells (Figure 1).

The vesiculo-vacuolar organelle

The VVO has been described and interpreted mainly using electron microscopy analyses, which have shown that VVOs are prominent structures in both tumor-supplying and normal vessel endothelial cells (11,12). Based on the use of various tracers, for example electron-dense ferritin, VVOs have been implicated as the primary pathway for macromolecular extravasation (9).

There is general consensus on the notion that vesicular transport across the endothelium (transcytosis) is an important mechanism for delivery of macromolecules to tissues. 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 at which the BBB is broken due to pericyte deficiency (13). 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

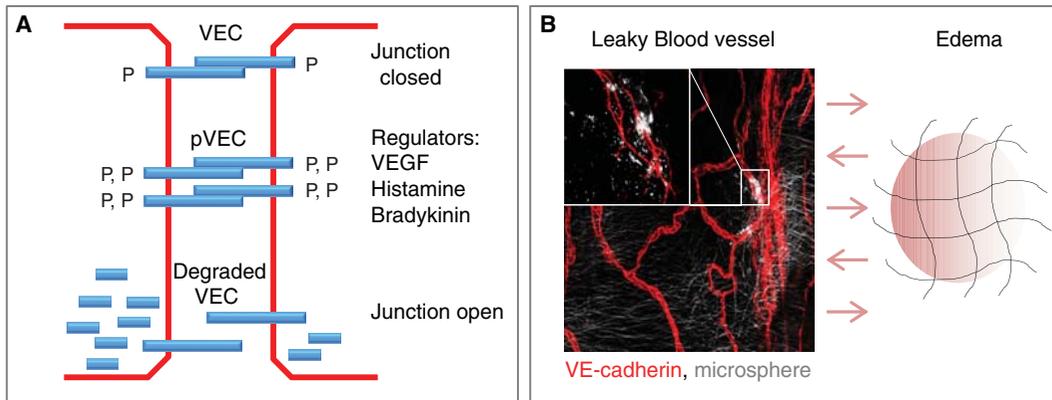


Figure 2. Opening of adherens junction in molecular extravasation. Panel A outlines schematically how VE-cadherin (VEC) engaged in hemophilic interactions at adherens junctions is regulated by hyperphosphorylation, correlating with internalization and degradation of VE-cadherin. VE-cadherin may also recycle; see text. Panel B shows leaky mouse tracheal vasculature after tail-vein injection of VEGF and fluorescent microspheres (white), followed by whole-mount immunostaining for VE-cadherin (red). VEGF-induced vascular leakiness leads to edema.

knock-out mice lacked caveolae and showed reduced permeability to macromolecules, the vasculature still contained VVOs (14). The origin of the VVO is therefore presently not known. A challenge in further analyses of VVOs is that they cannot be studied by conventional light microscopy. Moreover, there is at present no genetic loss-of-function model in which to study VVOs.

Paracellular junctions

Endothelial cell–cell junctions are organized into adherens and tight junctions. The main component of adherens junctions is vascular endothelial (VE)-cadherin, a transmembrane protein which forms homophilic complexes between endothelial cells (15). Adherens junctions dissolve in response to a number of stimuli, including vascular endothelial growth factors (VEGFs) and inflammatory cytokines such as histamine and bradykinin, to allow extravasation of macromolecules (see below). These stimuli cause VE-cadherin dissolution through a triggering event that may involve hyperphosphorylation of VE-cadherin (Figure 2). VE-cadherin is abundantly phosphorylated also in the basal, unstimulated state, through flow-mediated activation of c-Src, which triggers VE-cadherin phosphorylation directly or indirectly (16). The triggering event causing VE-cadherin internalization remains to be identified, and mechanisms different from a direct phosphorylation of VE-cadherin by c-Src have been suggested (17).

In a physiological setting, 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 (18). There are very

few reports showing junctions in their open state or the kinetics of opening and closure *in vivo* (19). In diseases characterized by excess vascular permeability (also denoted vascular leak), the regulation of junction dynamics is lost and the junctions remain open. This is denoted chronic permeability (20).

The actin cytoskeleton

Retraction of the endothelial cell body has been implicated in mediating increased vascular permeability (8). 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 (21,22). The role of the actin cytoskeleton needs to be further studied.

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 (6) for a detailed description. One example is the fenestrated endothelium that is present in vessels in endocrine glands, digestive tract mucosa, and the kidney peritubular capillaries. Here, endothelial cells are equipped with fenestrae, circular pores, covered by a diaphragm. A key protein in the diaphragm is plasmalemmal vesicle protein-1 (PV1), organized in radial fibrils. Gene targeting of PV1 does not prevent formation of fenestrae as such but results

in loss of the diaphragm and severe leakage of plasma proteins (23).

There are naturally occurring fenestrae without diaphragm, i.e. in the kidney glomerulus (6). The sinusoidal endothelium in the liver and the bone marrow also shows fenestrae without a diaphragm. These fenestrae, also denoted 'gaps', are heterogeneous but of larger diameter than the endocrine vessel fenestrae. How these large openings still maintain the vascular barrier is unclear.

The blood-brain barrier

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 (5). A potentially unique feature of the BBB is the trans-endothelial vesicular transport of a range of nutrients and metabolic waste products (24). There is 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 (see below) (25). Comparative information on molecular mechanisms in central nervous system and peripheral permeability is also lacking.

Vascular permeability to solute, molecules, and cells

Vascular permeability to solutes and small molecules occurs constitutively and appears not to require an active process. It is likely that the constant sieving of solute is important in maintaining the interstitial pressure in the tissue. It also serves to maintain the immune surveillance function of the lymphatics. Interstitial fluid collected by the lymphatics is carried via lymphatic capillaries to lymph nodes where foreign antigens will be exposed to the immune system (26).

Plasma contains three main molecular constituents: albumin, globulins, and fibrinogen (27). Extravasation of macromolecules serves diverse purposes, for example to maintain the balanced blood and interstitial pressures, to act in immune surveillance, 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 (28).

Extravasation of inflammatory and immune cells serves specific purposes in different pathologies. These cells are a prerequisite for healing of an acute disease process but may also propagate a chronic disease and interfere with recovery.

Overall, studies on the regulation of vascular permeability often suffer from the lack of physiological read-outs. It is clear, however, that permeability to solutes, molecules, and cells to some extent is differently regulated. Transient opening of paracellular junctions is the favored model for molecular extravasation. 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 unequivocally to sort out (29). Inflammatory cells adhere to the endothelium through binding to specific adhesion molecules on the endothelial surface. The cells can then transigrate directly through the thin endothelial wall, or pericellularly through endothelial junctions (30-32). 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 (33). Inflammatory cell extravasation was, however, not completely restricted. Indeed, the extent of immune cell extravasation appeared not to be affected (33). It is possible that different inflammatory cells extravasate through different mechanisms or that the cells are sufficiently plastic to adapt to the possibilities offered in the particular situation. Finally, exit of inflammatory cells may be differently regulated in acute and chronic inflammation.

Regulation of vascular permeability

Vascular permeability can be influenced directly by molecules that cause the barrier to disintegrate, whether it is a transvessel pore or a junction that needs to be opened. The relative extent of permeability can also be indirectly regulated by the blood pressure and the resulting blood flow. An increase in blood flow, e.g. as a consequence of vasodilation (34,35), will increase vascular permeability. Molecular regulators of vascular permeability include growth factors and inflammatory cytokines.

Angiogenic growth factors

VEGF was originally denoted vascular permeability factor (VPF) implying its essential role in regulation of the vascular barrier (36). VEGF is produced by all nucleated cells in the body; its expression is upregulated in hypoxia (37). VEGF binds to two structurally

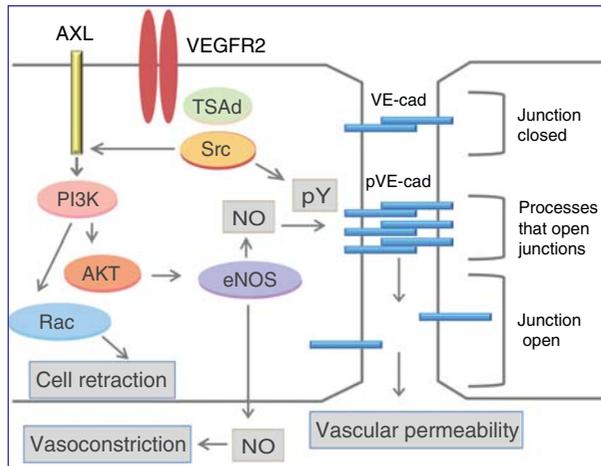


Figure 3. Signal transduction regulating opening of adherens junctions. Three main pathways are depicted: 1) VEGF-induced activation of c-Src leading to VE-cadherin (VE-cad) hyperphosphorylation (pY); 2) activation of eNOS leading to NO generation and effects on adherens junctions; and 3) activation of small GTPases such as RAC followed by rearrangement of the actin cytoskeleton and cell retraction. For details, see text.

related receptors denoted VEGFR1 and VEGFR2 (38). Both receptors, but preferentially VEGFR2, have been implicated in regulation of permeability and angiogenesis. The role of VEGFR1 is more unclear, and it may serve primarily as a negative regulator of VEGFR2. For details, see Koch et al. (38). Binding of VEGF to VEGFR2 leads to receptor dimerization, activation of the intracellular tyrosine kinase activity, and tyrosine phosphorylation of both the receptor itself and intracellular substrates for the kinase, so-called signal transducers. A number of phosphorylation sites in VEGFR2 have been identified (39). Several of these phosphorylation sites have been studied in loss-of-function analyses by phenylalanine knock-in, *in vivo* and/or *in vitro*. The most interesting site at this point appears to be the Y949 site in the VEGFR2 kinase insert. It serves as a binding site for an adaptor molecule, T cell-specific adaptor (TSAd), which binds to the cytoplasmic tyrosine kinase c-Src. Silencing or gene inactivation of TSAd makes endothelial junctions unresponsive to VEGF, resulting in loss of VEGF-induced vascular permeability (40). Several studies from the David Cheresh lab implicate c-Src in phosphorylation of the critical adherens junction protein VE-cadherin (41,42). According to the model, c-Src-induced phosphorylation of VE-cadherin promotes dissolution of VE-cadherin contacts between cells, followed by internalization and degradation or recycling of VE-cadherin (18). The other VEGFR2 phosphorylation sites induce signaling pathways that also contribute to vascular permeability regulation. These sites include

Y1173 (Y1175 in the human VEGFR2), which binds phospholipase C γ (43), as well as the adaptor molecule Shb (44), and Y1212 (Y1214 in the human VEGFR2), which binds the adaptor Nck (45). For details on their downstream pathways, the reader is referred to a previous study (46).

Whether other growth factors for which there are receptors on endothelial cells, such as placenta growth factor (binding exclusively to VEGFR1) or fibroblast growth factors (FGFs, binding to FGFR1 and FGFR2), mediate acute or chronic vascular permeability has not yet been addressed in detail.

Inflammatory cytokines

The two best-studied inflammatory cytokines in vascular permeability are histamine and bradykinin. Histamine is produced by mast cells and binds to G-protein coupled H1 and H2 histamine receptors (GPCRs) on endothelial cells (47). Bradykinin is cleaved from kininogen; it acts via GPCRs B1 and B2 (48). Although other mechanisms have not been excluded, it is quite well established that both histamine and bradykinin mediate activation of the serine/threonine kinase Akt, which phosphorylates and thereby activates endothelial nitric oxide synthase (eNOS) (49-52). Thereby, p-eNOS catalyzes the generation of NO. 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 (53). Akt is not the only kinase that can phosphorylate and activate eNOS, but it is the best-studied pathway. Another target effect of nitric oxide is S-nitrosylation of beta-catenin that will cause its dissociation from VE-cadherin and consequently the disassembly of adherens junctions (54).

The eNOS-NO pathway is implicated also in VEGF-regulated vascular permeability since ablation of eNOS expression blocks the VEGF response (51). Figure 3 depicts a schematic summary of signal transduction pathways regulating disassembly of adherens junctions.

Vascular permeability and disease

Vascular permeability and cancer

Tumor vasculature displays a spectrum of morphological and functional abnormalities including loss of vessel hierarchy, increased tortuosity, poor perfusion, instability, and increased vascular leakage (55). To a considerable extent, the tumor vessel phenotype is a consequence of hypoxia-driven persistent VEGF-production (3). Anti-angiogenic treatment e.g. using

VEGF-blocking antibodies or VEGFR kinase inhibitors therefore induces a more normal tumor vessel morphology and attenuates the exaggerated permeability (56). The therapeutic benefit of anti-angiogenic treatment in prolonging progression-free and overall survival depends on the cancer diagnosis, and I refer to in-depth recent reviews on this important matter (57). 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 permeability, or whether other effects of the treatment e.g. on neo-angiogenesis in the tumor are more important, has not been clarified.

The excess tumor vascular permeability has a range of deteriorating effects on the tumor microenvironment including increased interstitial pressure leading to impaired therapeutic delivery (58). 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 permeability 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 an acute increase in vascular permeability and tissue edema, impairing the ability of the heart to pump efficiently. Moreover, the increased permeability is manifested as increased infiltration of inflammatory cells in the acute phase after vessel occlusion (20,59). One of the first cell types to enter the infarcted myocardium is the neutrophil (60). 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 (NAPDH oxidase) and myeloperoxidase (MPO) (60). Elevated MPO levels predict the risk of heart disease in subgroups otherwise associated with low risk (61,62). Elevated MPO levels also independently predict the early risk of future cardiovascular events in patients with acute coronary syndromes (63,64).

Vascular permeability in retinal disease

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 (65). RCE cells possess intercellular tight junctions, which are formed by RCE and glial cells (66). Loss of normal BRB function is a common feature of many retinal

degenerative disorders including age-related macular degeneration, diabetic retinopathy, and retinal vein occlusions (67). Age-related macular degeneration patients present focal ischemia in the outer retina with associated inflammation, which induces VEGF production and angiogenesis resulting in hyper-permeable vessels. Prolonged elevation of blood sugar concentrations in diabetic patients causes endothelial apoptosis, basement membrane thickening, and pericyte loss, accompanied by increased VEGF synthesis and vascular permeability. Retinal vein occlusions can be attributed to hemodynamic disturbance (increased coagulation, impaired flow properties) resulting in ischemia and increased VEGF synthesis (see Stewart (67) for details). A common aspect of many eye diseases is therefore ischemia, increased VEGF production, and excess vascular permeability (68). The excess permeability has been attributed both to the overstimulated, abnormal vasculature and to changes in the phosphorylation of tight junction proteins such as occludin and zona occludens protein 1 (ZO1) (69).

Vascular permeability and lymphatics

The blood and lymphatic vasculatures constitute two parallel circulatory organs, connected by the emptying of lymph into the left jugular vein. Blind-ending lymphatic capillaries collect interstitial fluid by pumping the liquid, which will pass lymphatic valves that close to prevent 'back-flow'. Tissue edema facilitates the draining of the interstitial fluid through the initial lymphatic vessels by pulling on the vessels through their tissue-anchored filaments (70). In pathologies such as cancer, lymphatic vessels are often collapsed due to the excessive interstitial pressure and edema, implying that the lymphatic vasculature is dysfunctional (70). Several cell types in the cancer produce lymphatic growth factors, including VEGFC that binds the lymphatic receptor tyrosine kinase VEGF receptor 3 (VEGFR3) (71). Similar to the overstimulated and dysfunctional blood vasculature, the lymphatics may undergo neo-angiogenesis in cancer, which would facilitate draining of the tumor edema, on the one hand, but also, on the other hand, provide a route for spread of the cancer via the lymphatics. However, the relationship between vascular permeability and lymphatic function (collection and propagation of liquid, formation of new lymphatic vessels, and intra- and extravasation) is to a large extent unexplored.

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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