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Review

Do viral infections mimic bacterial sepsis? The role of microvascular permeability: A review of mechanisms and methods

B.E. Steinberg [a,1], N.M. Goldenberg [a,1], W.L. Lee [b,c,*]

[a] Department of Anesthesiology, University of Toronto, Ontario, Canada
[b] Keenan Research Center of the Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, Canada
[c] Division of Respirology and Interdepartmental Division of Critical Care Medicine, University of Toronto, Canada

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ABSTRACT

A dysregulated immune response and functional immunosuppression have been considered the major mechanisms of the bacterial sepsis syndrome. More recently, the loss of endothelial barrier function and resultant microvascular leak have been found to be a key determinant of the pathogenesis of bacterial sepsis. Whether a similar paradigm applies to systemic viral syndromes is not known. Answering this question has far-reaching implications for the development of future anti-viral therapeutic strategies. In this review, we provide an overview of the structure and function of the endothelium and how its barrier integrity is compromised in bacterial sepsis. The various *in vitro* and *in vivo* methodologies available to investigate vascular leak are reviewed. Emphasis is placed on the advantages and limitations of cell culture techniques, which represent the most commonly used methods. Within this context, we appraise recent studies of three viruses – hantavirus, human herpes virus 8 and dengue virus – that suggest microvascular leak may play a role in the pathogenesis of these viral infections. We conclude with a discussion of how endothelial barrier breakdown may occur in other viral infections such as H5N1 avian influenza virus.

Contents

1. Introduction

Bacterial sepsis, the syndrome of systemic inflammation following bacterial infection, is a clinical entity that is responsible for a large number of in-hospital deaths (Annane et al., 2003). An important but often unappreciated battleground in sepsis is the vascular endothelial barrier: both the local and systemic effects of infection and sepsis lead to a breakdown of microvascular barrier integrity. Increased efflux of fluid and macromolecules from the intravascular space results in profound tissue edema, hypotension and shock (Groeneveld et al., 1987; Riedemann et al., 2003; Russell, 2006) and likely contributes to the morbidity and mortality from sepsis

* Corresponding author. Tel.: +1 416 864 606077655.
E-mail address: warren.lee@utoronto.ca (W.L. Lee).
[1] These authors contributed equally.

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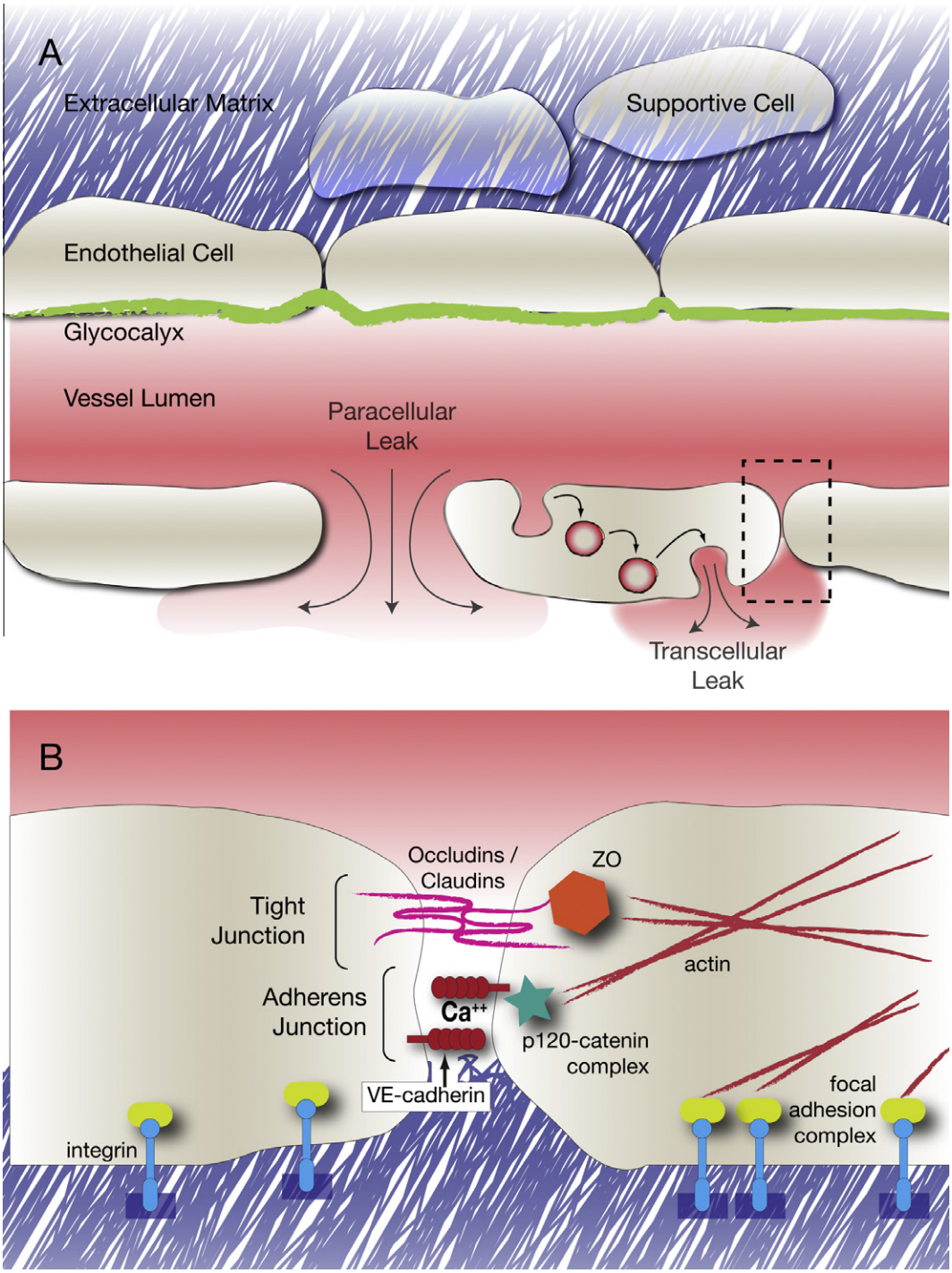

Fig. 1. Microvascular constituents and leak pathways. (A) The blood vessel lumen is lined by endothelial cells, which comprise the primary component of the microvascular permeability barrier. Vessel integrity is further influenced by the interaction of endothelial cells with the extracellular matrix, glycocalyx and supporting cells. In certain physiological and pathological settings, microvascular leak pathways can be initiated via paracellular and transcellular mechanisms as described in the text. (B) The principal inter-endothelial junctions are the tight junction and adherens junction, which interface with the cell's cytoskeleton via specific adaptor proteins. The tight junction is composed of claudin and occludin proteins, which interact with the zona occludens proteins (labeled ZO in the figure) along their cytoplasmic surface. The calcium-dependent homotypic interaction of endothelial VE-cadherin of the adherens junction is relayed to the actin cytoskeleton by multiple adaptor proteins. The p120-catenin complex is shown here. The importance of the different junctions in preventing leak varies across different vascular beds. The cytoskeleton also engages with integrin proteins that anchor endothelial cells to the surrounding extracellular matrix.

(Goldenberg et al., 2011). Whether a similar breakdown in vessel integrity occurs in systemic viral infections is unclear and forms the basis for this review.

We begin by introducing the principal components of the endothelial barrier and the pathways through which bacterial sepsis destabilizes them. We proceed by highlighting specific methods for studying vascular permeability in bacterial sepsis that may be of equal interest to viral researchers. An explicit appraisal of the benefits and limitations of the various *in vitro* and *in vivo* methods is included to help place the data into context. We then focus our

discussion on hantavirus, human herpes virus 8 and dengue virus as examples of viral infections that perturb the endothelial barrier. We conclude by discussing potential directions for future research.

2. Control mechanisms of endothelial permeability

The endothelial monolayer that lines all blood vessels functions to control the influx and efflux of materials between the vessel lumen and the interstitium. The movement of vessel contents into the interstitium can occur via paracellular and transcellular routes (Fig. 1A). Relatively little is known about the contribution of transcellular leak to vascular permeability during inflammation (Hu et al., 2008), and it is generally accepted that that the paracellular pathway is the dominant one (Aird, 2007).

The critical structural features in the post-capillary venule – the key site of inflammatory leak – include the adherens junction (AJ), the actin cytoskeleton, and several non-endothelial components, such as the basement membrane and glycocalyx (Aird, 2007; Marti-Carvajal et al., 2011) (Fig. 1A and B). Tight junctions (Gillrie et al., 2007; Montaner et al., 2003) are also present, although they contribute less to barrier integrity within the post-capillary venule than the AJ.

The predominant structural component of the AJ is vascular endothelial (VE) cadherin. Through VE-cadherin, neighboring endothelial cells are connected to one another in a calcium-dependent manner (Legrand et al., 2001). Additionally, the cytoplasmic tail of VE-cadherin interacts directly with β-catenin and p120-catenin to link the transmembrane protein to the actin cytoskeleton (Potter et al., 2005; Xiao et al., 2003). Phosphorylation of the intracellular domain of VE-cadherin regulates binding of β-catenin and p120-catenin (Potter et al., 2005). Binding of p120-catenin masks a dileucine motif on VE-cadherin, resulting in decreased clathrin-mediated endocytosis of VE-cadherin, stabilizing it at the AJ (Xiao et al., 2005). Furthermore, mutation of the intracellular domain of VE-cadherin does not affect the ability of VE-cadherin to bind neighboring cells, but greatly increases paracellular leak in cultured endothelial cells (Navarro et al., 1995). Mediators of inflammation, like VEGF and thrombin, signal through Src and Rac to trigger the serine phosphorylation of VE-cadherin. This leads to the endocytosis of VE-cadherin in a β-arrestin-dependent fashion (Gavard and Gutkind, 2006) (Fig. 2). Barrier-protective agents, such as angiopoietin-1 (Ang1) inhibit leak by blocking this pathway (Gavard et al., 2008).

One means by which the AJ acts to promote endothelial barrier function is through its linkage to the actin cytoskeleton. Actin-based structures are found in the spectrin skeleton beneath the plasma membrane, as well as in stress fibres, focal adhesions, and the cortical actin ring (Prasain and Stevens, 2009). Cortical actin is a functional ring of F-actin bundles that underlies the plasma membrane, tethered to various integral membrane proteins, such as VE-cadherin (Mehta and Malik, 2006; Sarai et al., 2009; Spindler et al., 2010). The polymerization and depolymerization of actin filaments, and the proportion of filaments devoted to various actin-containing structures is under tight regulation, and these mechanisms are largely beyond the scope of this review. However, one aspect that is particularly relevant to systemic inflammation is the role of the Rho superfamily of GTPases. The actin cytoskeleton is dynamically regulated by members of the Rho family of GTPases, notably Rac, Cdc42 and RhoA. Signaling through Rac1 and cdc42 results in the inhibition of cofilin and the recruitment of cortactin to stabilize cortical actin and enhance barrier stability (Kouklis et al., 2004; Lampugnani et al., 2002; Waschke et al., 2004). The endogenous lipid sphingosine-1-phosphate (S1P) decreases vascular permeability, in part, by stimulating endothelial Rac activity and increasing cortical actin (Fig. 2). In contrast, signaling through RhoA, which occurs in response to stimulation by thrombin and other inflammatory mediators (Breslin et al., 2006; Sun et al., 2006), destabilizes the endothelial junction and increases permeability. This is thought to occur via Rho kinase, which inactivates myosin light chain phosphatase, causing myosin contraction and destabilization of the actin cytoskeleton with subsequent reduction in VE-cadherin stability (Essler et al., 1998). Agents known to increase microvascular leak, such as thrombin, have long been observed to disrupt cortical actin and to initiate endothelial cell contraction by increasing the inward tension exerted by stress fibres (Dudek and Garcia, 2001; Hotulainen and Lappalainen, 2006). The balance between stress fibres and cortical actin is essential for normal endothelial barrier function, and factors influencing this balance may therefore represent

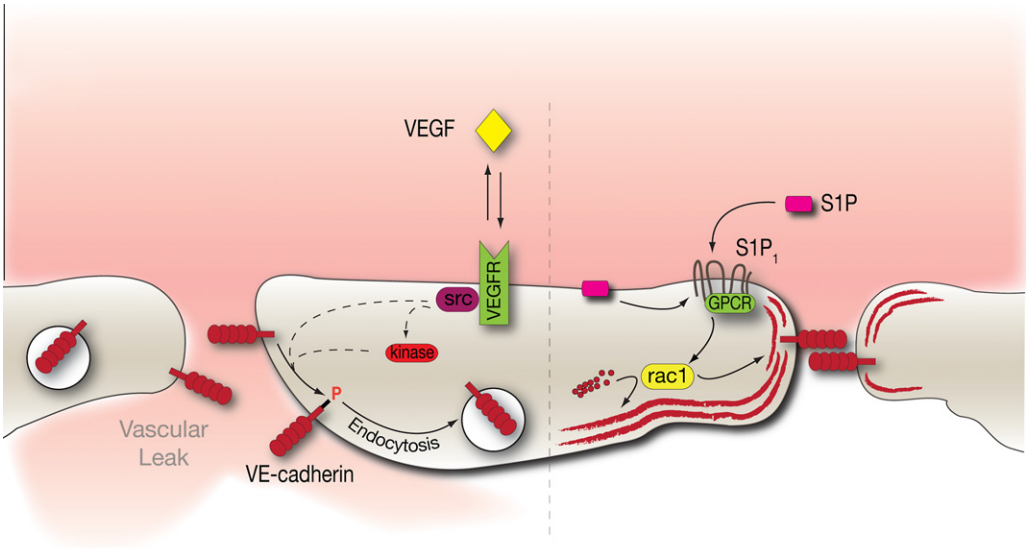

Fig. 2. Barrier disruptive and protective signaling pathways at the adherens junction. (Left) VEGF signaling through its receptor leads to the activation of src family kinases and downstream phosphorylation of the VE-cadherin, which is subsequently endocytosed. Removal of VE-cadherin from the inter-endothelial junction results in paracellular leak (Carr et al., 2003). Sphingosine-1-phosphate (S1P) is produced within endothelial cells as well as in the circulation. Binding to the S1P1 receptor (S1P1) leads to G-protein coupled receptor activation and downstream stabilization of the cortical actin network and maintenance of intercellular junctional proteins as described in the text.

important therapeutic targets. In particular, agents that promote cortical actin formation may have the potential of enhancing endothelial integrity.

In addition to the endothelial monolayer, several non-cellular components play an important role in establishing the microvascular permeability barrier. The luminal surface of the endothelium is lined by a complex mixture of plasma proteins, proteoglycans, and glycosaminoglycans collectively known as the glycocalyx (reviewed in (Mehta and Malik, 2006; Michel and Curry, 1999)). The strong negative charge of the glycocalyx electrostatically determines access of intravascular materials to the endothelial surface; disruption of this negative charge increases permeability to albumin and other macromolecules (Pries et al., 2000). Additionally, animal experiments have shown that intravenous administration of bacterial lipopolysaccharide results in degradation of the glycocalyx and increased microvascular leak (Marechal et al., 2008). Treatment of these animals with activated protein C (aPC) diminished leak while restoring glycocalyx morphology (Marechal et al., 2008).

Finally, complex interactions exist between endothelial cells and the extracellular matrix (ECM), but these are outside the scope of this review. Outstanding reviews on this topic have been published previously, such as the one by Mehta and Malik (2006). Importantly, the ECM provides both a scaffold for the endothelial monolayer as well as a rich source of extracellular signals that govern endothelial proliferation, migration and orientation. Release of ECM degradation products into the circulation is commonly seen in states of increased permeability, suggesting a role for the ECM in maintaining endothelial barrier integrity (Partridge et al., 1993; Resnikoff et al., 1999).

3. Changes in permeability in bacterial sepsis

The clinical syndrome of sepsis is characterized by the elevation of circulating inflammatory cytokines (reviewed in (Rittirsch et al., 2008)), dysregulation of coagulation (Stearns-Kurosawa et al., 2011), and may progress to profound tissue edema and shock, and unchecked endothelial activation (Shapiro et al., 2010). Many of these pathological changes converge at the endothelium. During sepsis, increased microvascular leak occurs due to some combination of endothelial damage (e.g. cellular apoptosis or necrosis), loss of intercellular junctional integrity, or remodeling of the cellular cytoskeleton. For instance, signaling through RhoA in response to VEGF, thrombin, LPS, and other inflammatory stimuli increases microvascular permeability (Breslin et al., 2006; Sun et al., 2006). Infection during sepsis can also increase leak *per se* through several different mechanisms; bacterial products like lipopolysaccharide can induce apoptosis in endothelial cells (Aliprantis et al., 1999; Bannerman and Goldblum, 2003). Additionally, toxin production following infection by *B. anthracis* or *C. sordellii* leads to aberrant signaling through the VE-cadherin cytoplasmic tail, which results in disassembly of junctions and increased vascular leak (Geny et al., 2007; Warfel et al., 2005). Finally, exciting work has also shown that an active form of the protein Slit, which signals through Robo4 to stabilize the adherens junction, improves survival in animal models of sepsis and H5N1 avian influenza (London et al., 2010).

Using endothelial monolayers, London and colleagues found that the active fragment of Slit, Slit2N, was able to decrease hyperpermeability as a result of TNF-α, IL-1β, or LPS stimulation (London et al., 2010). The mechanism by which this occurred was through the stabilization of VE-cadherin at the AJ. London's group then proceeded with a compelling series of *in vivo* experiments: Slit2N administration diminished endothelial leak in the mouse lung following the administration of intratracheal LPS, which mimics Gram negative sepsis (London et al., 2010). In a cecal ligation and puncture (CLP) model of polymicrobial sepsis, Slit2N treatment improved survival in mice. Finally, similar results were seen in mice infected with H5N1 avian influenza: Slit2N decreased endothelial hyperpermeability in the lung following viral infection (Fig. 3**)**. In all cases, the survival benefit occurred without any effect of Slit2N on cytokine levels (London et al., 2010). This supports the notion that the effect of Slit2N occurs at the endothelial level. Clearly the Slit-Robo4 axis represents a potential new avenue for therapy not only for sepsis, but for viral infections that result in breakdown of the microvascular barrier.

Another mechanism of endothelial leak is excessive endothelial activation. Recently the importance of endothelial NF-κB has been underscored by a series of elegant studies employing transgenic mice that conditionally over-express a degradation-resistant form of the NF-κB inhibitor I-κBα selectively in endothelial cells (Ye et al., 2008). In this model, the end result is that NF-κB is inhibited only in endothelium. In both endotoxemia and sepsis from cecal ligation and perforation, selective suppression of endothelial cell NF-κB improved multiple organ injury and survival without affecting the clearance of bacteria from the circulation, and without affecting levels of cytokines such as TNF-α, IL-1β, and IL-6 (Xu et al., 2010; Ye et al., 2008). How NF-κB suppression in endothelia resulted in improved survival is not clear; however, mice in which endothelial NF-κB was blocked exhibited decreased endothelial permeability (Ye et al., 2008). Other work has demonstrated the cytoprotective effects of diminishing NF-κB signaling, for example through the activation of the FLICE-like inhibitory protein (FLIP) in LPS-induced models of sepsis (Bannerman et al., 2004). As we indicate later, the NF-κB signaling pathway has also been implicated in H5N1 influenza (Schmolke et al., 2009) and HHV-8 infection (Bottero et al., 2011; Chugh et al., 2005; Konrad et al., 2009; Sadagopan et al., 2007; Sgarbanti et al., 2004).

Thus, it has recently become apparent that microvascular leak is a critical determinant of the pathogenesis of bacterial sepsis and a viable therapeutic target (Goldenberg et al., 2011). In retrospect, this was entirely consistent with the observation that the outcome during sepsis is largely determined not by the infectious agent itself, but by the host response to the infection (Gustot, 2011). Mortality remains high despite early antibiotic therapy, and patients with positive or negative cultures have comparable mortality rates following sepsis or septic shock (Brun-Buisson et al., 1995).

4. Methods for endothelial barrier investigation

Microvascular permeability is modulated by many parameters – the activation state of the endothelium, mechanical forces**,** pressure and chemical gradients, and extracellular organizational components like the glycocalyx – all of which are affected by physiological and pathophysiological processes. An appropriate experimental system is designed to explore a specific component while controlling the multiplicity of accessory processes and simulating *in situ* conditions. Researchers have approached this problem using model systems at three levels of increasing complexity. Cultured endothelial monolayers represent the most basic model, while intact animals are the most complex. Isolated micro-vessel systems work at an intermediate level.

In this section, we provide a brief review of a number of *in vitro* and *in vivo* methods that exist to study the impact of host–pathogen interactions on endothelial barrier function. As cell culture systems are the most prevalent in the literature, we include a discussion of their validity and limitations. Of note, the use of harvested primary endothelial cells can be fraught with many of the same limitations of immortalized cell cultures; specifically, primary endothelial cells partially de-differentiate from their endothelial phenotype once cultured without their specific *in situ* microenvironment (Cleaver and Melton, 2003).

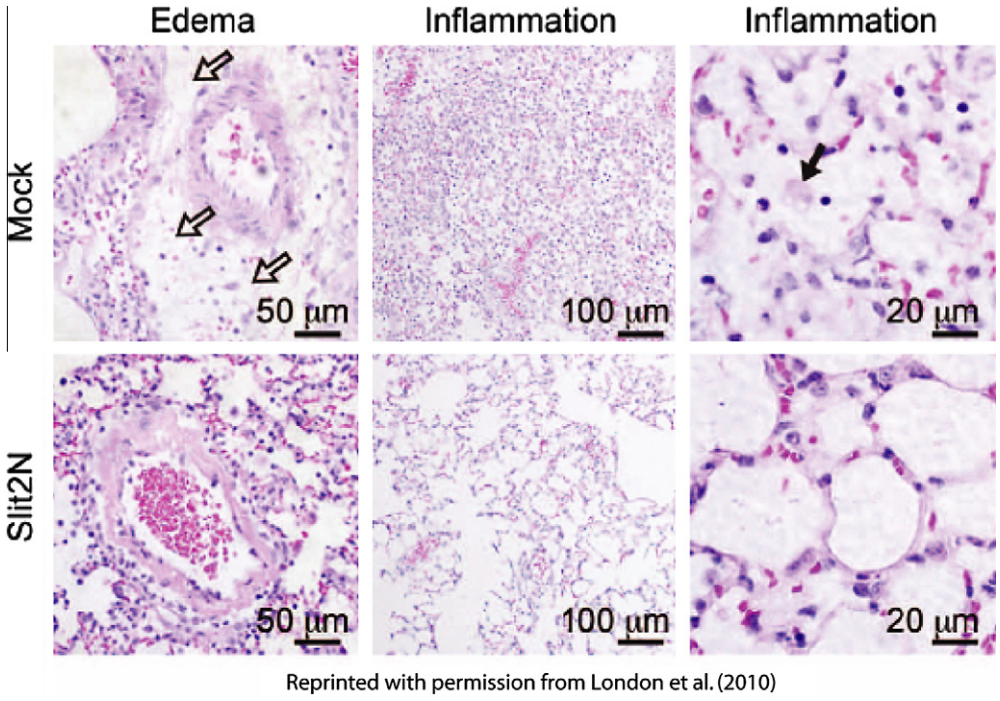

Fig. 3. Reproduced with permission from London et al. (2010). BALB/c mice were infected intranasally with H5N1 avian influenza virus and received intravenous Slit2N or Mock daily for 5 days. Mice were killed on day 6 after infection and H&E staining was performed on lung sections. White arrows show accumulation of edema fluid; the black arrow in the top right panel shows the presence of foamy macrophages.

4.1. In vitro methods

Perhaps the most commonly used method for assessing endothelial barrier integrity involves cultured endothelial monolayers grown on tissue culture well inserts (transwells). Two well-described assays are employed: in the first, the flux of large molecules across an endothelial monolayer is monitored. Commonly used substrates are labeled albumin or dextran. Dextran is a biologically inert molecule that has the advantage of being commercially available in a variety of polymer sizes, conjugated to a wide array of chromophores and fluorophores. It is relatively simple to accurately measure diffusion of fluorescent dextran across a cell layer into an adjacent chamber of culture medium under various growth conditions (i.e. in the presence or absence of different ions or growth factors).

In the second assay, an electrical current is used to measure the transendothelial electrical resistance (TEER) of cells grown on a transwell. For these measurements, the monolayer is treated as an impedance barrier, and a current is passed across the cell layer between an apical and basal culture medium chamber. The voltage across the monolayer allows for the calculation of TEER using Ohm's Law.

More recently, TEER measurements have been replaced by measurements of electric cell-substrate impedance sensing (ECIS) (Lo et al., 1995). In this method, cells are cultured directly on electrodes embedded within a culture plate. A current applied across the electrode is able to measure impedance over a given frequency range. By modeling the potential path of current across the cell layer, several parameters can be measured, including resistance of the monolayer, the cell thickness of the monolayer, and the capacitance of the different structural domains of the plasma membrane of individual cells in the monolayer (Lo et al., 1995). This method is capable of effectively measuring endothelial barrier function in real time (Fordjour and Harrington, 2009; Wang et al., 2010c).

A fourth use of *in vitro* monolayer-based assays is for microscopic studies. Endothelial monolayers provide a desirable platform for both live- and fixed-cell imaging of junctional structures and monolayer architecture using light and electron microscopy. Some laboratories have even been able to adapt this system for the *ex vivo* examination of patient endothelial cell samples (Fadini and Avogaro, 2010; Feng et al., 1999). While all of the *in vitro* (cell culture) assays have extensive limitations (discussed below), one key advantage is the ability to dissect out contributions from the direct effects of the virus on the endothelium as opposed to from the systemic inflammatory response.

A basic limitation of most endothelial cell culture models is that they do not accurately reflect the whole of the microvasculature. For instance, lack of shear stress and other cells (e.g. smooth muscle cells and pericytes) detract from the fidelity of the model. It is well known that cells cultured into monolayers have differing intercellular architecture to their *in situ* counterparts. This has been demonstrated by the observation of inter-endothelial gaps upwards of 5–10 μm within monolayers; *in situ* or in isolated microvessels, basal gaps are typically less than 1 μm (Baluk et al., 1997; Curry and Adamson, 2010; McDonald et al., 1999). In a similar manner, barrier-protective mechanisms observed in monolayer systems that produce closure of intercellular gaps through VE-cadherin remodeling have not been consistently observed *in situ*. For example, a pharmacologically induced increase in intracellular cAMP produced only marginal changes in baseline microvessel permeability without any significant observable enhancement in the plasma membrane VE-cadherin in perfused venules within rat mesenteries (Adamson et al., 2008). These data likely reflect baseline phenotypic differences between cultured and *in situ* endothelium.

Supportive structures within the microvascular unit, namely the glycocalyx, are not commonly considered in cell culture methods. It remains uncertain the extent to which the glycocalyx is structurally and functionally expressed in monolayers (Becker et al., 2010; Chappell et al., 2009; Gouverneur et al., 2006; Potter et al., 2009).

Physical and electrostatic repulsion by the glycocalyx influences plasma protein permeability, which may explain the observation that the permeability of rat and mammalian microvessels in some preparations is approximately 10-fold smaller than of cultured endothelial monolayers (Curry, 2005; Curry and Adamson, 2010; Michel and Curry, 1999). Vasoactive and inflammatory agents used to treat endothelial monolayers may also affect the function of other supportive cells *in situ*, including fibroblasts (Iversen et al., 2005; Reed and Rubin, 2010), lymphatics (Fox and von der Weid, 2002) and lymphocytes.

One of the major criticisms of cell culture models is the lack of flow, since shear stress is known to dramatically alter the phenotype of endothelial cells (Dai et al., 2004; Davies, 2009). Certain cell culture systems, like ECIS, can be modified to permit the addition of shear stress (DePaola et al., 2001; Levick and Michel, 2010). This limitation is nullified by microfluidic devices that allow for the investigation of whole vessels in the setting of physiologic shear stress (Gunther et al., 2010). In these assays, a whole vessel dissected from an animal is mounted and imaged in a physiological environment with flow through the vessel lumen and access to the extraluminal surface. Such experiments offer many intriguing possibilities; the vessel can be isolated from animals with different genetic backgrounds or disease states, and access to both the luminal and extravascular spaces makes microscopic and biophysical analysis of flow and macromolecule transport possible in an intact vessel. In principle, this would be a more physiologically accurate depiction of native vasculature than *in vitro* endothelial monolayers, since most vessel components are present at the time of assay, and mechanical stresses on the vessel will more closely mirror the *in vivo* environment. To date, this method has been used primarily for arterial vessels. However, it is likely that venular vessels could also be studied using the same system.

4.2. In vivo methods

One of the most basic and long-standing methods to investigate vessel permeability *in vivo* is the Miles assay, first developed to quantitatively assess vascular leak in the skin of guinea pigs (Miles and Miles, 1952). This assay measures a systematically injected tracer, such as Evans blue dye that binds non-covalently to albumin, which leaks into tissue beds. At a given time post-injection, the tissue can be homogenized, the dye extracted and quantified. Many adaptations have been described to evaluate accumulation in other organs like the lung and brain. In particular, some Miles assay adaptations use lectins that identify endothelial cells (McDonald et al., 1999; Thurston et al., 1996). These compounds are injected intravenously and followed by peroxidase or fluorescence detection to visualize lectin binding by fluorescence microscopy.

Miles assays have been used in a variety of virological studies. For examples, Evans blue was used in a hamster model of arenavirus infection (Gowen et al., 2010), rat models of respiratory syncytial virus infection (King et al., 2001; Piedimonte et al., 2000), and mouse models of West Nile virus infection (Morrey et al., 2008; Wang et al., 2008). In these studies, at a pre-determined post-inoculation time, a dose of Evans blue dye is administered and the organ of interest is examined for dye extravasation at autopsy.

The prevalence of this assay speaks to its relative simplicity. Nevertheless, there are several limitations to the Miles assay that merit consideration. Most significantly, vascular permeability in organ tissues is dependent not only on the status of the various endothelium permeability pathways, but concomitantly on the influence of vascular perfusion and microvasculature pressure (Bates, 2010). The Miles assay cannot distinguish between tracer that remains present within the vasculature from that which has in fact extravasated. This limitation can partially be overcome by employing a double tracer method (Nagy et al., 2008). In this protocol, an initial tracer is injected systemically. After a predetermined time period contingent on the specific experimental design, a second intravenous tracer is injected prior to collecting a blood sample and euthanizing the animal for tissue harvest. The second tracer now provides a measure of intravascular space, whereas the initial tracer estimates the combined intravascular and extravascular volumes. One important caveat to this method is that it assumes that even in the setting of a highly permeable microvasculature – as is the case in sepsis – the second intravenous tracer has extravasated to a negligible extent. Alternatively, many groups attempt to perfuse the animals to simply remove any intravascular tracer. Several laboratories have adapted these strategies in rat and mouse vasculature permeability studies (Curry et al., 2010; Nedrebo et al., 2003; Renkin and Tucker, 1998). These assays are terminal procedures, inherently limited to single interventions after which the animal is sacrificed at a specific time point. Prohibitively large cohorts may thus be required for adequate comparison between control and treated animals over time. The terminal nature of the method provides information on net accumulation while excluding acquisition of information surrounding dynamic fluctuations in vascular permeability.

Interestingly, longitudinal studies can be carried out using MRI-based approaches with high molecular weight contrast agents (Fu and Shen, 2004; Huxley et al., 2007) and serial imaging. This latter approach is similar to the use of albumin labeled with long-wavelength fluorophores using whole-animal fluorescence scanning methods (Kim et al., 2009). MRI can also monitor the formation and progression of tissue edema by processing T2-weighted images to quantitatively generate apparent diffusion coefficients of water (see (Paul et al., 2001) for example).

A final *in vivo* method is that of intravital microscopy. In this system, epifluorescence, confocal, or two-photon microscopy is used to visualize the microvasculature in living animals (Chappell et al., 2006; Kenne and Lindbom, 2011). This method allows for the direct visualization and quantification of molecular leak from vessels using fluorescently labeled tracers (Oschatz et al., 2011). Additionally, intravital microscopy can be used to observe the interaction between labeled cell types – for instance leukocytes and the endothelium – during different experimental conditions (Kempf et al., 2011). These methods provide meaningful information on their own and can also be used as *in vivo* adjuncts to simpler *in vitro* TEER or ECIS measurements.

5. Case studies

The central issue of this review is whether viral infections can cause endothelial leak akin to what occurs in bacterial sepsis. If this were to be the case, it would open up a new field for antiviral research. As indicated above, there is precedent: the use of Slit2N ameliorated vascular leak and improved survival in mouse models of bacterial sepsis (cecal ligation and perforation) as well as after infection with H5N1 avian flu (London et al., 2010). In the following section, we briefly review three different viruses whose pathogenesis is putatively mediated in part through interaction with the endothelium.

5.1. Hantavirus

Hantaviruses – including the Puumala, Hantaan, Sin Nombre and Andes viruses – are a group of RNA viruses that mainly infect capillary endothelial cells (Mackow and Gavrilovskaya, 2009). These pathogens are responsible for two main human illnesses: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), both of which present with edema and hemorrhage (for clinical features see (Chang et al., 2007; Mertz et al., 2006;

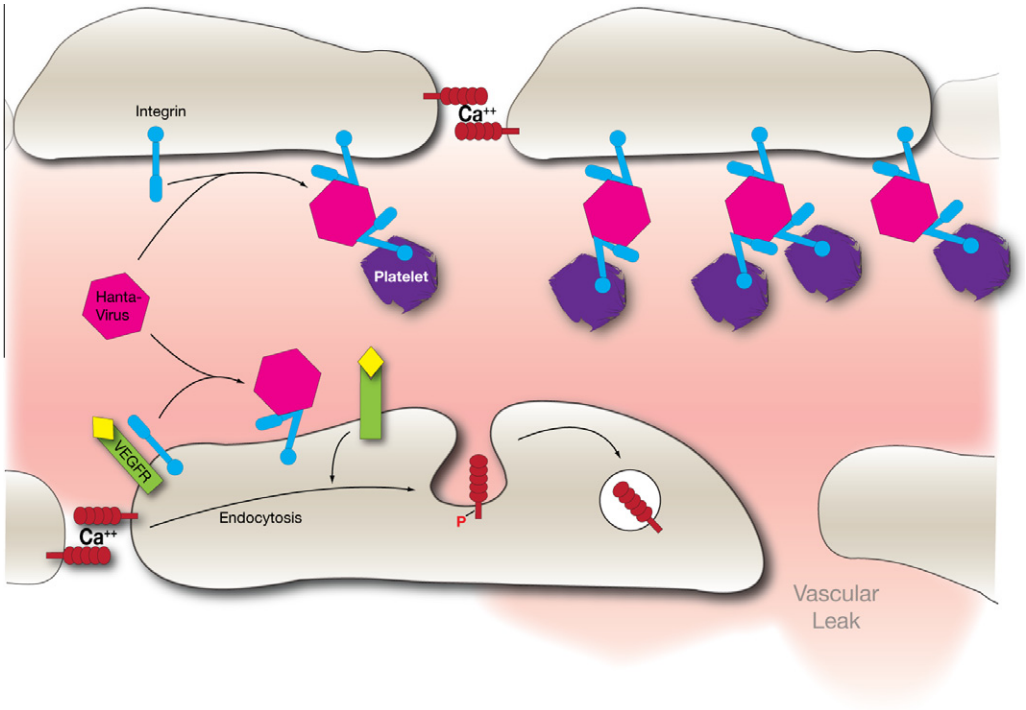

Fig. 4. Virus-mediated disruption of the endothelium microvasculature by Hantavirus. In the basal state, plasma membrane integrins reduce the influence of VEGFR on junctional VE-cadherin. Hantavirus engages these integrin receptors, thereby promoting VEGFR-mediated endocytosis of VE-cadherin (Gavrilovskaya et al., 2008; Gorbunova et al., 2010). The virus can also engage integrins on circulating platelets (Gavrilovskaya et al., 1998, 2010).

Stollenwerk et al., 2008)). In addition to affecting platelet count and activation, hantaviruses increase endothelial permeability without causing endothelial cell lysis (Wahl-Jensen et al., 2007; Yanagihara and Silverman, 1990; Zaki et al., 1995). This is primarily achieved by two mechanisms; disruption of integrin function and the internalization of VE-cadherin (Fig. 4). Traditionally, activated T cells were hypothesized to damage the endothelium, leading to increased permeability (see (Borges et al., 2006; Ennis et al., 1997; Terajima et al., 2007) for examples). For example, *in vitro* work by Hayasaka et al. (2007) demonstrated increased endothelial permeability when Sin Nombre-infected endothelial cells were exposed to hantavirus-specific cytotoxic T cells. However, these results only provide limited evidence of an immune-mediated mechanism of disease given the highly engineered nature of the experimental system. More recent compelling data support a direct effect of hantavirus on the pulmonary vascular endothelium in HPS: virus-infected hamsters develop the pulmonary syndrome even in the absence of T cells (Hammerbeck and Hooper, 2011) In both models of the disease however, microvascular barrier disruption plays a central role.

β_3 Integrins are ligands for hantavirus particles, and hantavirus infection yields a similar phenotype to β_3 integrin knock-out mice (Hodivala-Dilke et al., 1999). In a resting endothelial cell, β_3 integrins complex with the vascular endothelial growth factor (VEGF) receptor, VEGFR2 (Borges et al., 2000), and regulate VEGF signaling (Robinson et al., 2004). Hantavirus particles bind uniquely to an inactive conformation of β_3 integrins (Gavrilovskaya et al., 1998). As such, mice lacking β_3 integrin, or animals infected with pathogenic strains of hantavirus, display an increase in VEGF-induced endothelial permeability (Gavrilovskaya et al., 2008). Therapeutic potential lies in two barrier-stabilizing molecules – sphingosine 1-phosphate (S1P) and angiopoietin 1 (Ang1) – both of which block this pathological increase in endothelial permeability (Gavrilovskaya et al., 2008).

In addition to binding β_3 integrin on the endothelial cell surface, hantavirus also interacts with β_3 integrin on resting platelets (Gavrilovskaya et al., 1998, 2010). This has two deleterious effects: platelets are essentially cross-linked to infected endothelial cells by viral particles thus lowering the circulating platelet count, and platelet activation is inhibited due to the preferential interaction of hantavirus particles with inactive β_3 integrin (Gavrilovskaya et al., 1998, 2010). Together, these effects may play an important role in the hemorrhage that is characteristic of hantavirus infections.

The second major pathway by which hantavirus decreases endothelial barrier function is through disruption of VE-cadherin localization to adherens junctions. Pathogenic strains of the virus cause increased phosphorylation of VEGFR2 in infected endothelial cells (Gorbunova et al., 2011). This, in turn, leads to increased phosphorylation and internalization of VE-cadherin, resulting in increased microvascular leak (Gorbunova et al., 2011). An antibody that blocks VEGFR2 is able to block internalization of VE-cadherin in infected cells (Shrivastava-Ranjan et al., 2010). Similarly, *in vitro* treatment with pharmacologic inhibitors of VEGFR2 and Src kinase block increased permeability in Andes virus-induced endothelial leak models (Gorbunova et al., 2011). In these studies, endothelial cell monolayers were infected with virus and permeability was quantitatively assessed by the movement of fluorescently labeled dextran across the monolayer. S1P and Ang1 are both capable of preventing the aberrant targeting of VE-cadherin seen in hantavirus-infected cells (Gorbunova et al., 2011) and, as mentioned earlier, may have therapeutic potential in HPS and HFRS.

The investigation of hantavirus infection on the endothelium will benefit from the availability of the Syrian hamster HPS model (Hooper et al., 2001). This model system impressively reproduces many of the features of the human disease including similar incubation times and disease onset, pulmonary effusion and edema, hypotension and shock, thrombocytopenia and neutrophilia, and – germane to this discussion – endothelial cell infection (Campen et al., 2006; Hooper et al., 2001; Wahl-Jensen et al., 2007). The Syrian hamster model, amenable to the multiplicity of *in vivo* tech-

niques described above, may soon be complemented by the ability to follow pulmonary edema in live animals by chest radiography (Brining et al., 2010; D. Safronetz, personal communication). This technique may prove to be a cost-effective means of evaluating potential therapeutics. These include – but are certainly not limited to – treatment with Slit2N peptide similar to mouse models of H5N1 avian influenza infection (London et al., 2010). Similarly, pharmacological investigation of tyrosine kinase inhibitors, such as pazopanib (Gorbunova et al., 2011), as well as S1P and Ang1 signaling modulators may now have a tractable animal model for study.

5.2. Human herpes virus 8 (HHV-8)

HHV-8, also known as the Kaposi's sarcoma-associated herpes virus, is a human oncogenic γ-herpesvirus that predominantly infects endothelial and B-cells (Dupin et al., 1999; Flore et al., 1998). In the setting of immunosuppression, infection most commonly results in Kaposi sarcoma (KS), the second most frequent tumor in individuals with acquired immunodeficiency syndrome (Chang et al., 1994). Primary effusion lymphoma and multicentric Castleman's disease may also develop secondary to HHV-8 infection in immunosuppressed individuals (Cesarman and Mesri, 2007; Dupin et al., 1999; Oksenhendler et al., 2002).

Systemic vascular leak is not a notable clinical finding in these patients, yet pathologically KS displays an angioproliferative phenotype and may affect multiple organs including the skin, lungs, kidney, gastrointestinal tract, liver and mucosa. The HHV-8 G-protein coupled receptor (vGPCR) protein, encoded by the *ORF74* gene, is a key determinant; in fact, expression of the protein in endothelium alone recapitulates the formation and progression of KS-like lesions in mouse models (Grisotto et al., 2006; Montaner et al., 2003; Mutlu et al., 2007).

A recent study by Dwyer et al. has also implicated vGPCR as a concurrent modulator of endothelial barrier structure and integrity (Dwyer et al., 2011) (Fig. 5). Using *in vitro* and *in vivo* methodologies the authors demonstrated increased paracellular permeability in the setting of vGPCR expression. Specifically, they addressed the impact of HHV-8 on permeability employing trans-endothelial monolayer dextran permeability assays in conjunction with a modified Miles assay in mice with tumor allographs induced by immortalized endothelial cells with or without vGPCR expression. In ancillary experiments, the structural basis of their physiological studies was explored by imaging of endothelial monolayers with both light and electron microscopy. Their work demonstrates that by activating the PI(3)K-γ and Rac signaling pathways, vGPCR signaling increases VE-cadherin phosphorylation, endocytosis and ensuing junction destabilization (Dwyer et al., 2011). AJ remodeling and increased vascular permeability were confirmed in a panel of human KS biopsy samples. Notably, alterations in tight junctions were also observed (Dwyer et al., 2011). In the setting of bacterial sepsis, the AJ of post-venule capillaries are of primary importance; however, the relative importance of tight and adherens junction disruption in HHV-8 infection remains unclear.

Increases in VEGFR2 phosphorylation have been detected with stable vGPCR expression (Dwyer et al., 2011), and represent another potential contributory mechanism to vGPRC-induced increases in vascular permeability. Moreover, high levels of inflammatory cytokines and VEGF have been described in vGPCR-driven murine tumors (Ascherl et al., 1999; Martin et al., 2008; Montaner et al., 2004). These findings, in combination with the work of Dwyer et al. (2011), suggest that vGPCR is able to impair the vascular barrier both directly (vGPCR) and indirectly – via VEGF and other inflammatory mediators. The extent to which this barrier disruptive pathway contributes to the pathogenesis of KS has yet to be defined, but likely contributes at a minimum to the KS angioproliferative phenotype. The use of VEGF inhibitors (reviewed in (Bhargava and Robinson, 2011)) will afford the opportunity to examine VEGF's influence in HHV-8 pathology.

Lastly, HHV-8 modulates other cellular pathways, including MAPK, MMP-9 and HO-1 [see (Sahni, 2007) and references therein] in addition to the interaction of vGPCR with the endothelial barrier. The activation of MAPK pathways induces activator protein-1 and IL-6, both of which affect cellular proliferation and survival (Xie et al., 2005). MMP-9 and HO-1 expression are also increased during HHV-8 infection (Marinissen et al., 2006; McAllister et al., 2004; Wang et al., 2004). The induction of MMP-9 is notable as this member of zinc-dependent endopeptidases signals through VEGF

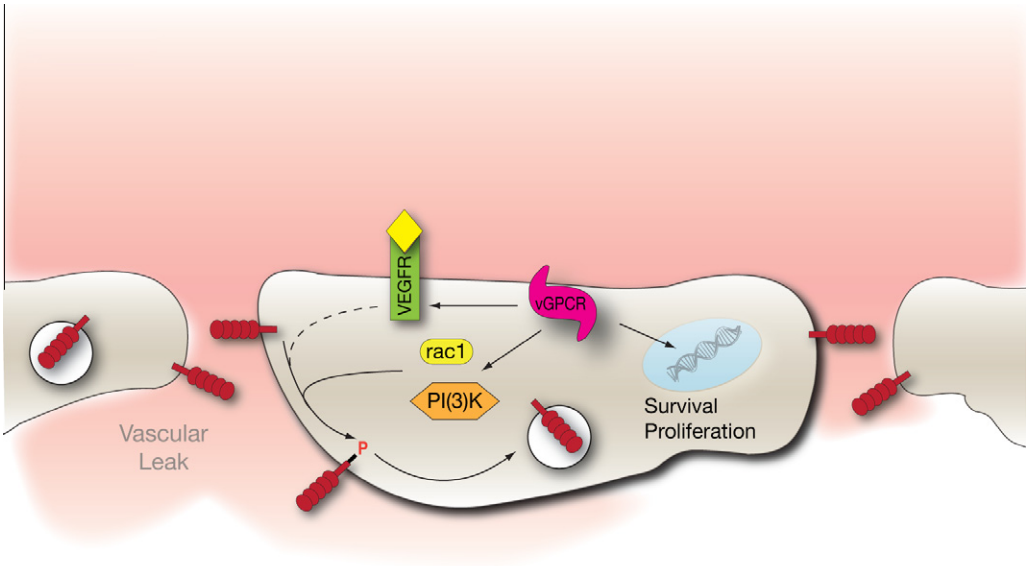

Fig. 5. Virus-mediated disruption of the endothelial microvasculature by vGPCR in HHV-8 infection. The HHV-8 viral protein vGPCR is the primary executor of the virus's barrier disruptive phenotype. Based on the recent work of Dwyer et al. (2011), the viral protein promotes VE-cadherin internalization and subsequent vascular leak by facilitating VEGFR activation as well as PI(3)K and Rac signaling pathways. The protein may concurrently activate endothelial cell survival and proliferation pathways (see main text and references therein for details).

receptors and may represent an important signal for tumorigenesis. HO-1 may contribute to the malignant phenotypic transformation induced by vGPCR and interference with its signaling has been suggested as a potential therapeutic strategy (Marinissen et al., 2006). All of these signaling pathways represent additional mechanisms by which the virus may directly and/or indirectly affect endothelial function; the relative importance of each remains to be clearly defined.

Endothelium infected with HHV-8 has been shown to undergo NF-κB activation, which promotes efficient viral replication (Chugh et al., 2005; Sgarbanti et al., 2004). As discussed above, endothelial NF-κB has been implicated in modulating the microvascular barrier at least in animal models of sepsis through a series of experiments employing endothelial-specific inhibition of NF-κB activity (Bannerman et al., 2004; Xu et al., 2010; Ye et al., 2008). Using this system, inhibition of endothelial NF-κB resulted in improved hemodynamic parameters, decreased tissue edema, and increased survival in endotoxemia and polymicrobial bacterial sepsis (Song et al., 2009; Xu et al., 2010; Ye et al., 2008). Whether HHV-8-induced activation of NF-κB is important for its disruption of barrier integrity is unknown. More critically, whether inhibition of HHV-8-mediated vascular leak *in vivo* changes the pathology or outcome is unknown. This is largely due to the lack of an appropriate animal model, although some progress is being made (Chang et al., 2009).

5.3. Dengue virus

Dengue virus (Nagy et al., 2008), an enveloped ssRNA virus of the *Flaviviridae* family, represents another example of an infection that affects endothelial barrier function. Its global footprint remains vast with an estimated 2.5 billion people at risk of infection and an annual incidence of 50 million (Halstead, 2007). The virus, of which four serotypes have been described, is composed of three structural and seven non-structural proteins and causes a wide spectrum of disease in humans, ranging from the relatively mild dengue fever to the severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The latter are characterized by abnormal hemostasis and increased vascular permeability. Interestingly, even in the setting of pleural, pericardial and peritoneal effusions, pathological studies of DHF and DSS show limited capillary damage (Limonta et al., 2007), implying that the loss of endothelial barrier function is not due to endothelial cell death. The use of human pathological samples has obvious limitations for the proposal of mechanisms of disease; only correlations can be inferred by this method.

DV infection has been shown to ultimately modulate cytoskeleton and junctional complexes (Talavera et al., 2004) and lead to increased endothelial cell culture monolayer permeability (Wang et al., 2010a) consistent with the clinical presentation of DHF and DSS. Multiple mechanisms likely contribute to the increased vascular leak, including endothelial cell activation (Fig. 6). In this model, immune cytokines (e.g. TNF-α) indirectly lead to barrier disruption (Carr et al., 2003; Dewi et al., 2004; Lee et al., 2006; Leong et al., 2007) as seen in individuals with bacterial sepsis. DV infection leads to decreased circulating levels of activated protein C (Cabello-Gutierrez et al., 2009), which may also contribute to the mechanism of vasculopathy in DHF and DSS (Cabello-Gutierrez et al., 2009) (Fig. 6). Other work implicates DV-infected dendritic cells that over-produce soluble gelatinolytic matrix metalloproteinases leading to decreased junctional VE-cadherin and cytoskeleton redistribution, and thereby potentially enhancing endothelial permeability (Luplertlop et al., 2006). These indirect modulators of endothelial integrity exert their effect in concert with the direct modulation of the endothelial cytoskeleton by DV.

Whether blocking vascular leak improves the outcome of DSS was addressed in an intriguing study in which BALB/c mice were infected with Dengue virus (strain P23085) intraperitoneally at a dose known to cause vascular leak and death (Groger et al., 2009). Three days after infection, mice were administered a fibrin-derived peptide ($B\beta_{15-42}$, also called FX06) intraperitoneally twice daily. This peptide fragment has previously been shown to bind VE-cadherin and decrease ischemia–reperfusion injury, although the molecular mechanism remains unclear. FX06-treated mice had significantly decreased lung and intestinal capillary leak and improved survival even though serum and tissue viral load was unchanged. The authors postulated that binding of FX06 to VE-cadherin leads to the dissociation of the src kinase Fyn from the VE-cadherin complex, causing Fyn to associate (and presumably phosphorylate) p190RhoGAP. This, they hypothesized, would lead to an inhibition of RhoA activity. To support their claims, they demonstrated that the peptide could inhibit thrombin-induced

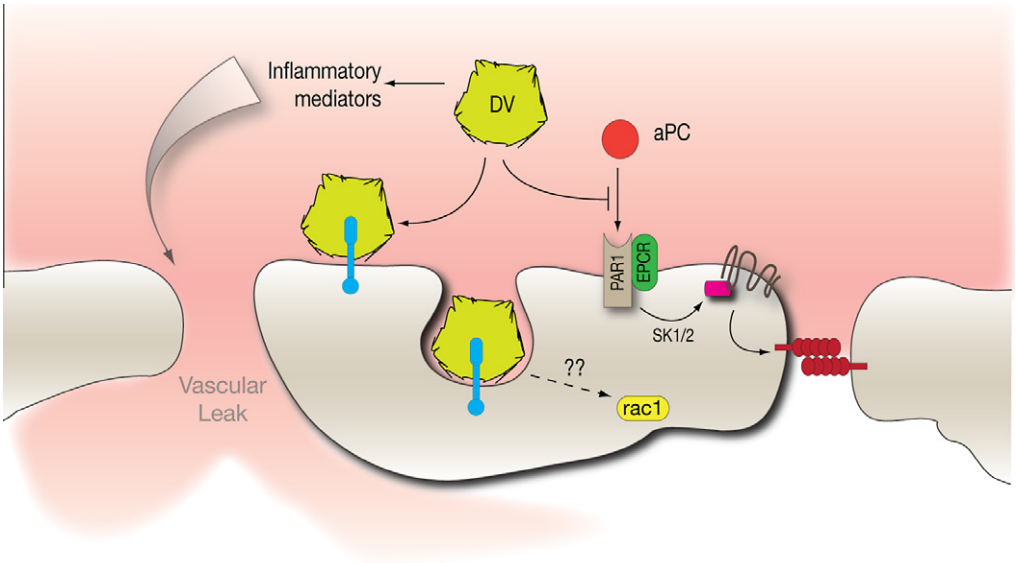

Fig. 6. Virus-mediated disruption of the endothelial microvasculature by Dengue virus. Dengue virus enters into cells by engaging plasma membrane receptors and inducing clathrin-mediated endocytosis (Krishnan et al., 2007). The systemic inflammatory responses initiated by the virus activate barrier-disruptive signaling (Carr et al., 2003; Dewi et al., 2004; Lee et al., 2006; Leong et al., 2007). Dengue virus infection also leads to decreased levels of circulating activated protein C (aPC) (Cabello-Gutierrez et al., 2009). aPC, when present, can bind its receptor on endothelial cells and initiate S1P production and stabilize the endothelium.

Table 1
Selected viral infections in which the endothelium has been implicated in the pathogenesis. Within the arenavirus and filovirus families, mechanistic differences in disease pathogenesis may exist between individual species. Notably, Lassa virus has been listed separately from other arenaviruses as it is known to have an atypical presentation compared with other viral hemorrhagic fevers [see (Kunz, 2009), for example]. For more details on filoviruses and arenaviruses, please consult the references listed.

Virus	Potential involvement of the endothelium in viral pathogenesis	Selected references
Dengue virus	- Disruption of cytoskeletal and inter-endothelial junctional complexes - Cytokine-induced endothelial cell activation - Decreased circulating of barrier-protective aPC	Cabello-Gutierrez et al. (2009), Tan et al. (2010), Wang et al. (2010a)
Hantavirus	- Increased vascular permeability without endothelial cell lysis via: - Disrupted integrin function including cross-linking platelets to endothelial cells via β_3 integrin - Increased VEGFR2 phosphorylation leading VE-cadherin internalization	Gavrilovskaya et al. (2008, 2010), Gorbunova et al. (2011), Shrivastava-Ranjan et al. (2010)
Human herpes virus 8	- Endothelial vGPCR expression recapitulates KS-like lesions in murine models - vGPCR-induced PI(3)K and Rac signaling and VEGFR2 phosphorylation initiate VE-cadherin phosphorylation and internalization - Endothelial NFκB activation promotes virus replication	Chugh et al. (2005), Dwyer et al. (2011), Mutlu et al. (2007)
Severe influenza	- Increased microvascular permeability via multiple mechanisms including systemic hypercytokinemia and increased programmed cell death - Endothelial barrier disruption countered by the Slit-Robo4 signaling axis - Endothelial cells function as key regulators of the systemic cytokine storm response and immune cell recruitment via S1P1 signaling pathway	London et al. (2010), Teijaro et al. (2011), Wang et al. (2010b)
Filoviruses (e.g. Ebola virus)	- Mucin domain from virion glycoprotein from Ebola can cause endothelial cell death and vascular leak *in vitro* - However, human autopsies and primate studies do not show prominent endothelial involvement - Microvascular leak is likely to result from systemic hypercytokinemia	Feldmann and Geisbert, 2011), Geisbert et al. (2003a,b), Stroher et al. (2001), Wahl-Jensen et al. (2005), Yang et al. (2000)
Lassa virus	- Endothelial cells can be infected and microvascular leak is associated with a poor prognosis. - Infection occurs via the α-dystroglycan receptor; replication is efficient but there is no cytopathic effect; thus, the mechanism of vascular leak is unclear - No significant cytokine storm compared to other viruses - Necropsy in primate models shows Lassa virus in endothelial cells of only terminal animals (i.e. not an early finding)	Cao et al. (1998), Hensley et al. (2011), Kunz, 2009), Lukashevich et al. (1999), Mahanty et al. (2001)
Non-lassa arenaviruses (e.g. Junin virus)	- Use transferrin receptor 1 as the cellular receptor; viral entry is not directly cytopathic - Virus infection (Junin) reduces expression of coagulation factors and increased prostaglandin PGI_2 and eNOS expression from the endothelium - Increased levels of systemic cytokines as compared with Lassa virus may contribute to endothelial involvement and disease pathogenesis	Gomez et al. (2003), Marta et al. (1999), Radoshitzky et al. (2007)

RhoA activation and that its vasculo-protective effect was lost in $Fyn^{-/-}$ mice. However, the authors did not measure RhoA activation after Dengue infection; more importantly they did not measure cytokine levels. Thus, an effect of FX06 on cytokines (e.g. TNF-α) and on non-endothelial cell types (e.g. leukocytes) cannot be excluded.

Recently, a non mouse-adapted strain of DV, termed D2Y98P, has been described which produces a clinical syndrome in susceptible mice that closely resembles the human disease spectrum (Tan et al., 2010). High-dose infection results in cytokine storm, vascular leak and hemorrhage, and rapid death, while low-dose infection causes non-paralytic death several days later (Tan et al., 2010). This feature should allow for more detailed analysis of the disease state in affected mice, and could potentially enable researchers to cross these mice with knock-out animals to dissect out pathways thought to be important in DV-induced disease. Furthermore, this model could represent an improvement over earlier models – such as that provided by Shresta et al. (2006) – due to this ability to control disease phenotype by adjusting viral dose. Importantly, the presence of viable animal models for this infection establishes the groundwork for the testing of hypotheses generated from cell culture experiments.

6. Conclusion

The importance of microvascular barrier disruption in the pathogenesis of bacterial sepsis is increasingly clear. Certain viral syndromes, HPS and DSS in particular, have clinical presentations strongly suggestive of a similar mechanism of disease.

The relative importance of endothelial leak in other viral infections is less certain. The H5N1 avian influenza strain that caused great concern a few years ago is known to produce marked edema and mortality in humans, although its infectivity remains low. The high mortality rate was attributed to a marked elevation of cytokines (cytokine storm) (Korteweg and Gu, 2008), which are well established to destabilize microvascular barrier integrity. Accordingly, there has been recent interest in whether the same phenomenon occurs with other strains of influenza, including the swine-origin H1N1 and seasonal influenza strains (Teijaro et al., 2011). Certainly, severe influenza infections are characterized by acute lung injury and, in some cases, severe hypotension necessitating treatment with vasopressors (Kumar et al., 2009). The mechanism of this effect has also been postulated to involve cytokines. For instance, infection of HUVEC in culture with seasonal influenza strains has recently been shown to lead to the production of tumor necrosis factor (TNF) α, interleukin (IL) 6, and IL-1β. These, in turn, have been postulated to upregulate host protease secretion with a resulting breakdown in inter-endothelial tight junctions (Wang et al., 2010b). While intriguing, such findings do not establish that infection with influenza leads to endothelial leak *in vivo*. First of all, HUVEC are not microvascular endothelial cells, in which adherens junction (rather than TJ) are thought to be the more relevant target. Furthermore, recent data cast doubt on the notion that swine-origin H1N1 influenza and seasonal influenza viruses lead to a cytokine storm (Woo et al., 2010). Instead, we postulate that viremia (as might occur in immunosuppressed patients or with virulent strains of the virus) can induce microvascular leak through other mechanisms. For example, work in one of our laboratories (manu-

script under review, W.L.L) suggests that human influenza can initiate programmed cell death within endothelial cells, which contributes to barrier breakdown.

Thus, viruses can cause microvascular leak either directly (through signaling or cytopathic effects) or indirectly, by inducing a systemic cytokinemia (e.g. H5N1). Obviously the relative contributions of these two pathways depend on the specific virus and the endothelial tissue bed (see Table 1). Nonetheless, the implication is that blocking microvascular leak may improve the outcome.

We are cognizant of the fact that much of the evidence describing the loss of endothelial barrier integrity in viral syndromes has come from cell culture models. Ultimately the relevance of these findings will be determined using animal models. In the meantime however, researchers can attempt to block endothelial permeability in cell culture using a variety of barrier-protective agents that have been described (Goldenberg et al., 2011), taking advantage of the ease with which specific molecules can be manipulated *in vitro*. For instance, it would be important and relatively simple to establish whether Angiopoietin-1 or Slit can prevent virus-induced endothelial leak and to determine the molecular determinants of this effect (e.g. VE-cadherin). Such experiments would lead naturally into therapeutic trials in infected animals that aim to improve survival.

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