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Role of Vascular and Lymphatic Endothelial Cells in Hantavirus Pulmonary Syndrome Suggests Targeted Therapeutic Approaches

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Abstract

Background: Hantaviruses in the Americas cause a highly lethal acute pulmonary edema termed hantavirus pulmonary syndrome (HPS). Hantaviruses nonlytically infect microvascular and lymphatic endothelial cells and cause dramatic changes in barrier functions without disrupting the endothelium. Hantaviruses cause changes in the function of infected endothelial cells that normally regulate fluid barrier functions. The endothelium of arteries, veins, and lymphatic vessels are unique and central to the function of vast pulmonary capillary beds that regulate pulmonary fluid accumulation.
Results: We have found that HPS-causing hantaviruses alter vascular barrier functions of microvascular and lymphatic endothelial cells by altering receptor and signaling pathway responses that serve to permit fluid tissue influx and clear tissue edema. Infection of the endothelium provides several mechanisms for hantaviruses to cause acute pulmonary edema, as well as potential therapeutic targets for reducing the severity of HPS disease.
Conclusions: Here we discuss interactions of HPS-causing hantaviruses with the endothelium, roles for unique lymphatic endothelial responses in HPS, and therapeutic targeting of the endothelium as a means of reducing the severity of HPS disease.

Introduction

The vasculature is constantly exposed to viral pathogens yet only a few viruses specifically target the endothelial cell (EC) lining of vessels and cause acute edematous or hemorrhagic disease. Hantaviruses predominantly infect the endothelial cell lining of vessels and nonlytically cause two diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS).[1–13] The mechanisms by which hantaviruses disrupt fluid barrier integrity and clearance functions of the endothelium are beginning to be disclosed and appear to involve dysregulating EC functions that normally restrict fluid leakage from vessels and clear fluid from tissues.[6,14–20]

Capillaries, veins, and lymphatic vessels are lined by a single layer of ECs that collectively form one of the largest tissues of the body.[21,22] The endothelium forms a primary fluid barrier within vessels but serves as more than just a conduit for blood to reach and return from tissues.[21,23] The endothelium selectively restricts blood and plasma from entering tissues, regulates immune cell infiltration, and responds to damage by limiting leakage, repairing vessels, and directing angiogenesis.[21] These ubiquitous functions require the endothelium to respond to a host of systemic and locally generated factors that alter inter-endothelial cell adherence and fluid barrier properties. Consequently, capillary barrier integrity is redundantly regulated by an array of EC-specific effectors that coordinately balance vascular fluid containment with tissue-specific needs, and respond to a host of systemic and locally generated factors that alter inter-endothelial cell adherens junctions.[21,24–32] ECs respond to activated platelets and immune cells, clotting cascades, chemokines and cytokines, growth factors, nitric oxide, and hypoxic conditions.[21,27,33–35] However, ECs also secrete cytokines, complement, and growth factors that positively or negatively impact the adherence and activation of platelets and immune cells, regulate responses to hypoxia, and restrict fluid accumulation in tissues.[21,23,25,34,36–38] Each of these EC responses is controlled by intertwined sensors and signals aimed at returning the endothelium to a resting state, countering permeabilizing effectors, repairing vessel damage, and restoring fluid and oxygenation levels within tissues.[21,24,39–44]

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The unique endothelium of capillaries, veins, and lymphatic vessels is central to their discrete fluid barrier and clearance functions.[36,45–47] Nonlytic viral infection of microvascular or lymphatic ECs (MECs, LECs) may disengage one or more fluid barrier regulatory mechanisms, thereby increasing vascular leakage or fluid clearance and as a consequence result in tissue edema.[48–52] However, the accumulation of interstitial fluids can result from either increased endothelial permeability or decreased lymphatic vessel clearance of tissue fluids. Altering LEC responses results in decreased lymphatic vessels clearance functions and lymphedema.[36,46,47,53] In the lung, lymphatic vessels clear fluid influx from interstitial spaces and keep pulmonary alveolar spaces relatively dry to permit gas exchange.[36,46,47,53] Failure of lymphatic vessels to clear fluids has spawned interest in the role of unique LEC and lymphatic vessel functions and regulation that contribute to edematous disease.

Vascular permeability induced by nonlytic viruses is likely to be multifactorial in nature, resulting from virally altered EC responses and signaling pathways, tissue hypoxia, immune cell and platelet functions, and a collaboration of dysregulated interactions that bypass redundant systems which control normal fluid barrier functions.[14–17,19,54] Failure of the endothelium to regulate fluid accumulation in tissues has severe pathologic consequences, and during HPS results in localized vascular permeability and acute pulmonary edema that contribute to cardiopulmonary insufficiency and a ~40% mortality rate.[4–6,9] The mechanisms by which HPS causing hantaviruses induce vascular permeability and acute edema following infection of ECs remains be defined. Recent clues to the role of vascular and lymphatic EC functions suggest potential therapeutic mechanisms that may stabilize the endothelium.

Hantavirus Infection and Disease

Hantaviruses are enveloped, tripartite, negative-sense RNA viruses and the only members of the *Bunyaviridae* that are directly transmitted to humans by mammalian hosts.[13,55,56] The hantavirus genome consists of three segments denoted S, M, and L, based on the length of their RNA segments, respectively.[13] The L segment encodes the 250 kDa RNA dependent RNA polymerase.[13,55] The S segment encodes a 48 kDa nucleocapsid (N) protein, which is the most abundant hantavirus antigen present in infected cells.[13] The M segment encodes two viral surface glycoproteins Gn (64 kDa) and Gc (54 kDa) that are co-translationally cleaved and targeted to the ER/cis-Golgi.[13,57] Hantaviruses bud internally into the lumen of the cis-Golgi and exit cells via a secretory mechanism consistent with aberrant vesicular trafficking.[13] Hantaviruses are both released from ECs and remain cell-associated through interactions with cell surface receptors.[14,54,58] GnGc heterodimers on the virion surface are presumed to bind cellular receptors and mediate viral entry into cells.[13,14,19,57–64]

In vitro, hantaviruses replicate to low titers, with initial viral progeny emerging from infected ECs 18–24 hours post infection (hpi), and $\sim 5 \times 10^6$ maximal titers days after infection.[13] Infection of ECs is nonlytic and the permeability of infected EC monolayers is not increased by infection alone.[13,15,65] Prototypic HPS (SNV, ANDV, NY-1V), HFRS (Hantaan virus-HTNV),[3,5,10,66,67] and nonpathogenic (Tula virus-TULV and Prospect Hill virus-PHV)[68–70] hantaviruses all infect human ECs regardless of their ability to cause disease, suggesting that EC entry alone is not a cause of pathogenesis.[12,15,71] At least two requirements for hantaviruses to be pathogenic have been determined thus far, the ability of hantaviruses to regulate early interferon responses and the use of specific integrins by pathogenic (ANDV, SNV, NY-1V, PUUV, SEOV, HTNV) but not nonpathogenic (PHV, TULV) hantaviruses.[19,61,62,64,72–75]

At least 17 hantaviruses cause HPS, also termed hantavirus cardiopulmonary syndrome (HCPS). Sin Nombre (SNV) in North America and Andes (ANDV) in South America[4–9,76–79] are prototype HPS viruses that cause acute pulmonary edema, cardiopulmonary insufficiency, and ~35%–40% mortality rates.[4–9,76–78,80–84] One to two weeks after infection, rapid onset of pulmonary edema and hypoxia occurs 6–12 hours after cough and rapidly progresses in severity.[4–6] Bilateral pulmonary infiltrates may be interstitial or alveolar with large pleural effusions of 2–8 liters at necropsy resulting from pulmonary edema fluid accumulating at up to 1 liter per hour in SNV patients.[44–6,8,9]

Hantaviruses are one of only a few viruses that primarily infect the EC lining of the vasculature.[8,9,12,71,85] Hantavirus antigen is found predominately in vast pulmonary capillary EC beds but is present in ECs within lymph nodes and throughout the body.[8,9,85] However, cytopathic effects are not evident following hantavirus infection of ECs *in vitro* or *in vivo*.[9,15,65] Histologically, the heart, kidneys, brain, and adrenals are grossly normal, with pulmonary alveoli filled with acellular proteinaceous fluid, yet the alveolar epithelium remains intact.[4–6,8,9] The most striking HPS findings are edematous lungs with up to 8 liters of pleural edema.[5,6,8,9] Pulmonary edema fluid contains few leukocytes, is largely serous in nature, and is consistent with the nearly complete loss of an alveolar capillary fluid barrier.[4–6,9] The absence of disrupted endothelium during HPS is similar to edematous pulmonary responses observed in patients with high altitude induced pulmonary edema (HAPE).[49,86] The long prodrome and rapid onset of edematous symptoms late after hantavirus infection[6] suggests the importance of targeting vessel stability in regulating HPS edema.

Vascular and Lymphatic Endothelium: Control of Vascular Fluid Barrier Functions

The endothelium is a remarkable tissue that functions to meet tissue needs, repair vascular damage, and restrict infection. The endothelium lines a series of discrete vessel types that conduct fluids to and from tissues, direct the transfer of nutrients, wastes, and oxygen, and coordinate tissue responses to changing conditions and pathogens.[21,47,52,87–89] Vascular ECs serve mainly as a conduit in the lining of high pressure arteries but take on a variety of fluid and cellular barrier functions in low pressure veins and capillaries that innervate organs and tissues.[47] Lymphatic vessels have a primary role in draining fluid, proteins, and immune cells from tissues and returning these components to the venous circulation.[36,45–47] Depending on their location, lymphatic vessels serve discrete fluid barrier and regulatory functions, keeping pulmonary alveolar spaces dry and clearing fluid influx from the lungs.[47,90,91] These diverse EC settings require discrete EC functions to effect exchange within large capillary beds of the kidney, liver, and lung.[47]

The endothelial fluid barrier is primarily derived from unique adherens junctions (AJs) composed of an EC-specific vascular-endothelial cadherin (VE-cadherin).[24,28,38,89.92–94] EC barrier functions are increased by the presence of cell surface presented VE-cadherin and reduced by the dissociation and internalization of VE-cadherin.[24,28,89] Phosphorylated VE-cadherin is internalized by its interaction with intracellular actin complexes, and this process is regulated by a variety of cellular receptors and intracellular signaling pathways. VE-cadherin phosphorylation is downregulated by an EC-specific phosphatase (VE-phosphatase) and several pathways that either directly or indirectly induce AJ assembly and EC integrity by returning VE-cadherin to an unphosphorylated resting state.[24,89] Chemokines, cytokines, and growth factors indirectly act on EC adherens junctions to increase vascular permeability and thus have the potential to contribute to pathogenic vascular leakage.[27,92]

Unique VEGF Receptors Regulate Capillary and Lymphatic Function

Vessel-specific ECs contain unique VEGF receptors 1/2/3 (VEGFR1/2/3) that respond to novel forms of VEGF (VEGF A-E) and positively or negatively impact AJ stability and vascular integrity.[91,92,95] VEGF-A binds to EC-specific VEGFR2 receptors and activates a Src-Rac-Pak-VE-cadherin pathway resulting in AJ disassembly and vascular permeability.[24,28,89] LECs uniquely express VEGFR3 on their surfaces and respond to VEGF-C/D, but also co-express VEGF-A-responsive VEGFR2 receptors and are further regulated by the formation of VEGFR2/3 heterodimers.[33,36,46]

A second EC-specific growth factor, angiopoietin-1 (Ang-1), binds to Tie-2 receptors and transdominantly blocks VEGF-A directed permeability. Ang-1 activates an alternate signaling pathway that inhibits VEGFR2 directed Src pathway responses and stabilizes VE-cadherin assembly into AJs. Sphingosine-1-phosphate is a lipid mediator produced by platelets that also stabilizes vessels by engaging Edg-1 receptors on ECs and blocks VEGF-A-directed permeability.[15,31,39,43,136,139]

VEGF-A was originally discovered as a potent vascular permeability factor that induces acute edema.[27,96] VEGF-A reportedly acts within 0.5 mm of its release,[97] and circulating soluble VEGFRs prevent VEGF-A from systemically permeabilizing vessels.[33,96] VEGF-A is induced by hypoxia, and reduced oxygen levels cause HAPE.[34,98,99] This results from activating the hypoxia-induced transcription factor-1a (HIF-1a), which senses oxygen levels and transcriptionally induces VEGF-A.[52,100–102] VEGF-A further upregulates HIF-1α, forming an autocrine loop which amplifies hypoxia-mediated VEGF- responses and EC permeability during HAPE.[52,86,98,103] Although this response fosters increased gas exchange, in continued low oxygen environments, these cellular responses instead cause pulmonary edema and respiratory distress.[98,101] These findings indicate that EC responses control capillary leakage through interconnected mechanisms and suggest that altering any number of orchestrated EC barrier functions can result in edema.

Pathogenic Hantavirus Binding to Inactive $\alpha_v\beta_3$ Integrins Regulates EC Permeability

Several studies demonstrate that monolayers of hantavirus-infected ECs are not permeabilized by infection alone[14,15,65] and instead indicate that pathogenic hantavirus infected ECs are hyperpermeabilized by VEGF-A.[15] The cellular entry of pathogenic hantaviruses is dependent on the presence of $\alpha_v\beta_3$ integrins on human ECs, while nonpathogenic hantaviruses PHV and TULV use $\alpha_5\beta_1$ integrins.[61,62] Pathogenic hantaviruses bind to inactive basal conformations of $\alpha_v\beta_3$ integrin receptors on ECs while nonpathogenic hantaviruses interact with discrete integrins.[61,62,64] Receptor binding directs viral entry, but at late times post-infection cell-associated hantaviruses also negatively impact $\alpha_v\beta_3$ integrin functions.[14–17,19,54] Days after infection, cell-associated pathogenic hantaviruses block $\alpha_v\beta_3$ integrin directed EC migration and direct the binding of quiescent platelets to the EC surface.[14,54]

Curiously, $\alpha_v\beta_3$ integrins present on ECs normally regulate vascular permeability. Ectodomains of $\alpha_v\beta_3$ and VEGFR2 form complexes that direct EC migration, a process that requires AJ disassembly, yet need to limit VEGF-A induced permeability.[92,104] Knocking out β_3 integrins or antagonizing $\alpha_v\beta_3$ results in enhanced VEGF-A directed permeability of capillaries *in vivo* and *in vitro*.[105–107] Inhibiting β_3 integrin functions causes vascular permeability disorders.[92,107–110]

Similar to antagonizing or knocking out $\alpha_v\beta_3$ integrins,[92,107] pathogenic hantavirus infection sensitizes ECs to the permeabilizing effects of VEGF-A.[15,16] SNV-, ANDV-, and HTNV-infected ECs, but not nonpathogenic PHV or TULV-infected ECs, are hyperresponsive to the permeabilizing effects of VEGF-A,[15] and VEGFR2 is hyperphosphorylated following pathogenic hantavirus infection.[14,17] Enhanced permeability of infected ECs only occurs days after infection when cell-associated hantaviruses coat the EC surface and inactivate $\alpha_v\beta_3$ integrins.[14–16,19,58] These findings, in the context of hypoxic HPS patients, suggest that hantavirus binding to inactive $\alpha_v\beta_3$ integrins contributes to capillary permeability in HPS. These results further suggest a mechanism for hantavirus-enhanced EC permeability that stems from disrupting normal $\alpha_v\beta_3$-VEGFR2 interactions and enhanced VEGFR2-Src-VE-cadherin signaling responses that dissociate VE-cadherin from AJs.[14–16,19,24,92] Collectively, these findings demonstrate that cell surface hantaviruses alter normal EC functions that control VEGF-A directed vascular permeability.[14–17,54,111]

Hantavirus–Endothelial Edemagenic Mechanisms

Prominent pulmonary dysfunction is a component of HPS disease and likely stems from hantavirus infection of ECs that line vast alveolar and renal capillary beds.[4–6,8,9,112,113] Acute pulmonary edema is a hallmark of HPS, and HPS patients arrive at hospitals in acute respiratory distress.[4] During HPS, bilateral pulmonary fluid infiltrates accumulate at up to a liter per hour, resulting in respiratory insufficiency and patient hypoxia during a critical phase of the disease.[4,6,8,9] The cause of acute edema following hantavirus infection is likely to revolve around the ability of the hantaviruses to infect ECs within alveolar capillary beds that normally regulate edema and gas exchange within the lung. Pathogenic mechanisms accounting for this extraordinary rate of pulmonary fluid accumulation have yet to be defined, but are likely to be multifactorial and require both capillary leakage and decreased pulmonary fluid clearance by lymphatic vessels.[6,15,16,114–116]

Clues to the mechanism of hantavirus-induced edema come from disparate findings on the role of hypoxia in acute pulmonary edema, the role of $\alpha_v\beta_3$ and VEGFR2 EC responses

described above. Recent studies of ANDV-infected LECs further demonstrate that normal EC VEGFR2/3 signaling responses are impaired by pathogenic hantaviruses.[6,14,15,19,114] Hypoxia is a prominent component of HPS patients which directs VEGF-A secretion.[5,6,8,9] Hypoxia-induced VEGF-A leading to acute pulmonary edema may contribute to both vascular leakage and reduced lymphatic vessel fluid clearance.[116] In fact, HPS patient VEGF-A levels were markedly elevated in pulmonary edema fluid and PBMCs in acute early phases of HPS.[114] Although a demonstrated role for hypoxia in hantavirus-induced permeability has yet to be conclusively defined, the ability of extracorporal membrane oxygenation (ECMO) to reduce HPS patient mortality[4,6] strongly suggests a role for hypoxia and VEGF-A in the acute pulmonary edema of HPS patients.

Potential Role of Hantavirus-Infected LECs in HPS Edema

Pulmonary lymphatic vessels are responsible for clearing fluid from alveoli and providing a semi-dry state that permits gas exchange.[45,47] Failure of lymphatic vessels to clear fluids results in lymphedema and suggests an additional mechanism for hantavirus-infected LECs to contribute to acute pulmonary edema during HPS.[36,46,47] Analysis of pathology samples from HPS patients indicates that hantavirus antigen is present in LECs of patient lymph nodes.[8,9,85] Although less is known about LECs, as described above, LECs express unique cell surface receptors and their integrity is regulated by both VEGF-A and VEGF-C.[36,46,47,53] Interestingly, LEC VEGFR3 receptors respond to VEGF-C and are associated with reduced tissue edema,[36,53] while inhibiting VEGFR3 signaling results in lymphedema.[36]

Our recent study indicates that ANDV infects LECs, alters LEC barrier functions, and causes the formation of giant LECs that are likely to alter lymphatic vessel functions.[116] However, the role of lymphatic vessels and LEC responses remains to be investigated in HPS patients. ANDV infection of LECs could alter lymphatic vessel responses to VEGF-A/C that contribute to pulmonary edema and HPS. Consistent with this, ANDV-infected LECs resulted in their hyperpermeability in response to VEGF-A that is blocked by VEGF-C. Unexpectedly, it was also found that ~70% of ANDV-infected LECs were giant cells (viable, 4–5 × normal size). Giant cells are caused by mutations in tuberous sclerosis complex (TSC1/2) proteins resulting in activation of mTOR (mammalian target of rapamycin).[117–126] Further, VEGFR2 responses activate mTOR, while rapamycin inhibits giant cell and VEGF-A permeability responses.[117,119,120,127,128]

Following ANDV infection of LECs, both giant cell and permeability responses were inhibited by rapamycin or VEGF-C, suggesting that ANDV alters normal hypoxia-VEGFR2-mTOR signaling pathways of LECs.[116] Hypoxia induces VEGF-A/Akt/mTOR signaling but these responses are offset by hypoxia-induced REDD1 expression and the ability of REDD1 to stabilize TSC1/TSC2 complexes and block mTOR activation.[117,120,122,124,129–132] These findings suggest that ANDV selectively disengages normal hypoxia–VEGF-A responses that control mTOR activation, and instead activate mTOR signaling responses causing giant LECs and LEC dysfunction that contributes to HPS. These findings suggest the potential for targeting pathway specific LEC responses using VEGF-C or rapamycin as a means to regulate ANDV-induced lymphatic dysfunction and reduce pulmonary edema during HPS. The ability of rapamycin and VEGF-C to regulate VEGF-A directed lymphatic vessel responses suggests their potential as HPS therapeutics that may be applicable to additional causes of acute pulmonary edema.

Animal HPS Model

Only ANDV infection of Syrian hamsters (*Mesocricetus auratus*) serves as a model of hantavirus pathogenesis that mimics human HPS in onset symptoms and lethal acute respiratory disease.[18,133,134] Inoculation of Syrian hamsters with ANDV, but not SNV or other HPS causing hantaviruses, induces pathology approximating human disease. ANDV causes a fatal infection of Syrian hamsters with an LD_{50} of 8 plaque-forming units. The disease is characterized by large pleural effusions, congested lungs, and interstitial pneumonitis in the absence of disrupted endothelium.[18,133,134] The onset of pulmonary edema coincides with a rapid increase in viremia on day 6, and large inclusion bodies and vacuoles in ultrastructural studies of infected pulmonary ECs.[133,134] Viral antigen was localized to capillary ECs, alveolar macrophages, and splenic follicular marginal zones populated by dendritic cells. Interestingly, depletion of CD4 and CD8 T-cells had no effect on the onset, course, symptoms, or outcome of ANDV infection and indicates the absence of T-cell responses.[18] Consistent with the potential involvement of β_3 integrins and VEGF-A in this process, ANDV binds to conserved residues within PSI domains of both human and hamster β_3 integrins.[19,64] The role of lymphatic ECs and responses remain unstudied in the animal model or in HPS patient tissues but may be keys to resolving and reducing the severity of HPS disease. Thus, the mechanism of pathogenesis caused by ANDV is consistent with hypoxia–VEGF-A directed acute pulmonary edema that occurs in the absence of T-cell mediated pathology and may include lymphatic vessel dysfunction.[18] Studies in Syrian hamsters provide a means of defining determinants of ANDV pathogenesis and the evaluation of therapeutics that target barrier functions of the vascular and lymphatic endothelium.

Targeted Therapeutic Approaches for Stabilizing the Endothelium

Currently there are no effective therapeutics for hantavirus infections or disease. Antiviral effects of interferon or the nucleoside analog ribavirin are only effective prophylactically or at very early times post-infection.[13,135] They appear to target early viral replication but neither is effective 1–2 weeks post-infection after the onset of HPS symptoms.[4–6,135] An alternative approach against viruses with a long disease onset may be to target the acute pathologic response therapeutically instead of viral replication. Since hantaviruses infect and alter fluid barrier functions of the endothelium, targeting EC responses that transiently stabilize the vasculature has the potential to reduce the severity and mortality of HPS.[43,136,137] This approach also has the advantage of being implemented at the onset of symptoms where antiviral approaches appear to be ineffective.[135]

Antibody to VEGFR2 reportedly suppresses VEGF-A induced pulmonary edema and suggests the potential of therapeutically antagonizing VEGFR2-Src-VE-cadherin signaling

pathways as a means of reducing acute pulmonary edema during HPS.[17,24,43] Several well-studied VEGFR2 and Src inhibitors are in human clinical trials or are used therapeutically to treat human cancers and have the potential to reduce the severity of viral permeability based diseases.[17,36,43] *In vitro*, angiopoietin-1 (Ang-1), sphingosine-1-phosphate (S1P), pazopanib, and dasatinib inhibited EC permeability directed by pathogenic hantaviruses.[15,17] VEGFR2-Src signaling inhibitors as well as the S1P analog FTY720 are already in clinical trials or used clinically for other purposes.[31,139] The ability of rapamycin to block VEGF-A induced microvascular permeability further suggests its potential as an HPS inhibitor through changes induced in both capillaries and lymphatic vessels.[122,128,140] VEGF-C may similarly be used along or in conjunction with other inhibitors to target lymphatic EC dysfunction and enhance fluid clearance from HPS patient tissues.

Targeting EC responses provides a potential means of stabilizing HPS patient vessels and reducing edema. Endothelial cells present a plethora of targets that may regulate virally induced vascular permeability and lymphatic functions. Many compounds targeting pathways which stabilize interendothelial cell adherens junctions are already clinically approved for other indications. Targeting vascular and lymphatic endothelial responses may also be broadly applicable to reducing the severity of a wide range of viral infections that impact the endothelium and cause edematous diseases.

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