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Hantavirus interferon regulation and virulence determinants

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ABSTRACT

Hantaviruses predominantly replicate in primary human endothelial cells and cause 2 diseases characterized by altered barrier functions of vascular endothelium. Most hantaviruses restrict the early induction of interferon-β (IFNβ) and interferon stimulated genes (ISGs) within human endothelial cells to permit their successful replication. PHV fails to regulate IFN induction within human endothelial cells which self-limits PHV replication and its potential as a human pathogen. These findings, and the altered regulation of endothelial cell barrier functions by pathogenic hantaviruses, suggest that virulence is determined by the ability of hantaviruses to alter key signaling pathways within human endothelial cells. Our findings indicate that the Gn protein from ANDV, but not PHV, inhibits TBK1 directed ISRE, kB and IFNβ induction through virulence determinants in the Gn cytoplasmic tail (GnT) that inhibit TBK1 directed IRF3 phosphorylation. Further studies indicate that in response to hypoxia induced VEGF, ANDV infection enhances the permeability and adherens junction internalization of microvascular and lymphatic endothelial cells. These hypoxia/VEGF directed responses are rapamycin sensitive and directed by mTOR signaling pathways. These results demonstrate the presence of at least two hantavirus virulence determinants that act on endothelial cell signaling pathways: one that regulates antiviral IFN signaling responses, and a second that enhances normal hypoxia–VEGF–mTOR signaling pathways to facilitate endothelial cell permeability. These findings suggest signaling pathways as potential targets for therapeutic regulation of vascular deficits that contribute to hantavirus diseases and viral protein targets for attenuating pathogenic hantaviruses.

1. Introduction

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) (Duchin et al., 1994; Lahdevirta et al., 1982; Lee, 1982; Nichol et al., 1993; Schmaljohn, 2001; Yanagihara and Silverman, 1990; Zaki et al., 1995). HFRS results from infection by Eurasian hantaviruses (Hantaan virus, HTNV; Dobrava virus, DOBV; Puumala virus, PUUV) (Lahdevirta et al., 1982; Lee et al., 1982; Schmaljohn, 2001) while hantaviruses identified throughout the Americas (*i.e.* Andes virus, ANDV; Sin Nombre virus, SNV; New York virus, NYV) are associated with HPS (Duchin et al., 1994; Enria et al., 1996; Lopez et al., 1996; Nichol et al., 1993; Schmaljohn, 2001). In contrast, Tula virus (TULV) and Prospect Hill virus (PHV) are hantaviruses that are not associated with any human disease (Plyusnin et al., 1994; Yanagihara et al., 1987). While TULV and PHV differ from pathogenic hantaviruses by their use of discrete integrin receptors (Gavrilovskaya et al., 1998, 1999; Raymond et al., 2005), PHV also fails to regulate early IFN induction which restricts its replication in endothelial cells and likely contributes to its inability to be a human pathogen (Alff et al., 2006, 2008; Geimonen et al., 2002; Spiropoulou et al., 2007). These findings suggest that hantaviruses contain virulence determinants that restrict antiviral IFN pathway signaling responses and alter normal endothelial cell signaling pathways that control vascular permeability.

Only a few viruses specifically target the endothelial cell (EC) lining of vessels and cause acute edematous or hemorrhagic disease. Mechanisms by which hantaviruses disrupt fluid barrier integrity and clearance functions of the endothelium are just beginning to be disclosed. Vascular permeability induced by hantaviruses is likely to be multifactorial in nature and result from virally altered EC responses and signaling pathways, tissue hypoxia and immune cell and platelet functions (Gavrilovskaya et al., 2010, 2012a,b,c, 2013; Gorbunova et al., 2010, 2011, 2013; Hammerbeck and Hooper, 2011; Kilpatrick et al., 2004; Koster and Mackow, 2012; Mori et al., 1999; Raymond et al., 2005; Taylor et al., 2013; Terajima et al., 1999; Vaheri et al., 2013). This is likely to occur through a collaboration of interactions which bypass redundant vascular systems that control critical fluid barrier functions. Failure of the endothelium to

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regulate hemorrhage or edematous fluid accumulation in tissues has severe pathologic consequences. Deficits in the regulation of vascular permeability are dramatically illustrated by findings in HPS patients which result in localized acute pulmonary edema, unprecedented pulmonary fluid accumulation rates (up to 1 l/h) and a ~40% mortality rate (Duchin et al., 1994; Koster and Mackow, 2012; Zaki et al., 1995). As the multifactorial nature of vascular regulation is impacted by many systems, a variety of hypotheses have been expounded, but need to be prefaced by stating that there is currently no data demonstrating that any of these theories play a causal role in vascular permeability induced by hantaviruses.

The primary understanding of hantavirus induced vascular deficits, remains the viruses ability to infect the endothelial cell lining of the vasculature and nonlytically cause edematous or hemorrhagic disease (Duchin et al., 1994; Lahdevirta et al., 1982; Lee, 1982; Nichol et al., 1993; Schmaljohn, 2001; Yanagihara and Silverman, 1990; Zaki et al., 1995). Hantaviruses dysregulate microvascular and lymphatic endothelial cell (MEC and LEC) functions that normally restrict fluid leakage from vessels and clear fluid from tissues (Gavrilovskaya et al., 2008, 2010, 2012c, 2013; Gorbunova et al., 2010, 2011, 2013; Koster and Mackow, 2012; Raymond et al., 2005; Shrivastava-Ranjan et al., 2010). The effects of hantavirus infection of endothelial cells remains enigmatic and the focus of our studies of altered endothelial cell signaling pathways (Gavrilovskaya et al., 2013; Gorbunova et al., 2011) that are fundamental to altered vascular permeability and hantavirus virulence.

Hantavirus entry into human endothelial cells initially discriminates between pathogenic hantaviruses, whose infection is fostered by human $\alpha_v\beta_3$ integrins, and nonpathogenic TULV and PHV which are unaffected by the presence of $\alpha_v\beta_3$ integrins (Gavrilovskaya et al., 1998, 1999; Raymond et al., 2005). Since $\alpha_v\beta_3$ is a known regulator of vascular permeability this finding ties hantavirus receptor usage to vascular permeability (Gavrilovskaya et al., 2008; Raymond et al., 2005). Yet, *in vitro*, pulmonary microvascular and lymphatic endothelial cells (MECs, LECs), and human umbilical vein endothelial cells (HUVECs) are not permeablized by hantavirus infection alone suggesting that receptor usage itself is not a cause of vascular permeability (Gavrilovskaya et al., 2008, 2012c, 2013; Gorbunova et al., 2010, 2013). Interestingly, studies indicating that cell-associated pathogenic hantaviruses bind inactive $\alpha_v\beta_3$ integrins, days after infection, tie $\alpha_v\beta_3$ integrin usage to the regulation of signaling pathways induced by a potent vascular permeability inducer, vascular endothelial growth factor (VEGF) (Gavrilovskaya et al., 1999, 2010; Raymond et al., 2005; Robinson et al., 2004). $\alpha_v\beta_3$ normally forms a complex with VEGF receptor 2 (VEGFR2), which tempers VEGFR2 directed permeability in response to localized VEGF release. Knocking out β_3 or inhibiting $\alpha_v\beta_3$ promotes VEGFR2 directed endothelial cell permeability (Borges et al., 2000; Byzova et al., 2000; Hodivala-Dilke et al., 1999; Reynolds et al., 2002; Robinson et al., 2004). Furthering this association during hantavirus infection, the permeability of endothelial cells infected by ANDV, SNV and NY-1V, but not nonpathogenic TULV or PHV hantaviruses, is dramatically enhanced in response to VEGF (Gavrilovskaya et al., 2008, 2010, 2012c; Gorbunova et al., 2010, 2011, 2013).

VEGF is a potent vascular permeability factor (VPF) that locally induces vascular permeability by binding endothelial cell VEGF receptors, within 1.5 mm of its release, and directing the disassembly of inter-endothelial cell adherens junctions (Dejana et al., 2008; Dvorak et al., 1995; Gavard, 2009; Gavard and Gutkind, 2006). VEGF is induced by hypoxia to facilitate repair, and increase gas exchange within the lung, and VEGF is inactivated by circulating soluble receptors that prevent systemic vascular permeability responses. VEGF induced pulmonary edema is known to be caused by hypoxia in high altitude settings (Berger et al., 2005; Hanaoka et al., 2003; Hopkins et al., 2005; Voelkel, 2002).

HPS patients are acutely hypoxic (Bustamante et al., 1997; Zaki et al., 1995) and a recent retrospective analysis of pulmonary edema fluids in a small number of HPS patients indicated the presence of high levels of VEGF (Gavrilovskaya et al., 2012a). Hantavirus infection of MECs and LECs may disengage one or more fluid barrier regulatory mechanisms, thereby increasing vascular leakage or fluid clearance resulting in tissue edema (Dehler et al., 2006; Schraufnagel et al., 2003). These findings suggest one of many mechanisms that may participate in HPS directed pulmonary edema and vascular deficits within hantavirus patients. However, although HPS patients are hypoxic there is as yet no causal evidence for this mechanism in hantavirus disease.

Consistent with roles for $\alpha_v\beta_3$ and hypoxia directed VEGF in hantavirus pathogenesis, hypoxia and VEGF tie into complex intracellular signaling pathways and feedback regulatory mechanisms that may be altered by virulence determinants within pathogenic hantaviruses. Hypoxia and VEGFR2 are tied to mTOR (mammalian target of rapamycin) directed cell division, control of cell size and feedback regulation of hypoxic responses (Kim et al., 2009; Xue et al., 2009). Studies presented below tie virulence determinants within hantavirus proteins to altered VEGF directed mTOR activation.

In addition to regulating cell receptor signaling, hantaviruses regulate IFN signaling pathways within human endothelial cells in order to successfully replicate and be human pathogens. Hantavirus replication is highly sensitive to the early addition of IFN or IFN pretreatment and hantaviruses grow to much lower titers in IFN competent cell lines than IFN locus defective Vero E6 cells (Alff et al., 2006). Interestingly, the effects of IFN addition are nearly absent when IFN is added 1 day post-infection (Alff et al., 2006), and consistent with hantaviruses inducing high level ISG responses at late times post-infection (Geimonen et al., 2002). In contrast to pathogenic hantaviruses, PHV rapidly induces IFNβ and IFN stimulated gene (ISG) responses that restrict its replication in human endothelial cells (Geimonen et al., 2002) and this response, in addition to receptor usage, are potential explanations for the absence of PHV associated human disease (Alff et al., 2006, 2008; Matthys et al., 2011; Matthys and Mackow, 2012). Our findings suggest that permissive hantavirus replication in human endothelial cells results from the selective restriction of early IFN induction (Alff et al., 2006; Geimonen et al., 2002; Matthys et al., 2011; Matthys and Mackow, 2012).

The ability of hantaviruses to regulate IFN induction and alter vascular and lymphatic endothelial cell signaling responses suggests the presence of encoded virulence determinants that permit viral replication and alter cellular responses which control fluid barrier functions of the endothelium. Here we show that hantaviruses contain virulence determinants that alter normal endothelial cell functions by regulating VEGF-mTOR signaling responses and permitting viral replication by inhibiting the early induction of Type 1 IFN. These findings suggest the presence of an IFN regulating virulence determinant in the Gn protein that is required for hantavirus replication in human endothelial cells and for subsequent vascular permeability deficits in HFRS and HPS patients. However, these clues to vascular dysfunction provide potential mechanisms by which hantaviruses induce vascular permeability and acute edema that remain to be defined *in vivo*.

2. Results

2.1. Hantavirus regulation of early IFN responses defines virulence determinants in the GnT

Replicating RNA viruses generate small amounts of dsRNA that are detected by cytoplasmic helicases which signal TBK1/IKKε