



Short Communication

Slit2-Robo4 receptor responses inhibit ANDV directed permeability of human lung microvascular endothelial cells

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ABSTRACT

Hantaviruses nonlytically infect human endothelial cells (ECs) and cause edematous and hemorrhagic diseases. Andes virus (ANDV) causes hantavirus pulmonary syndrome (HPS), and Hantaan virus (HTNV) causes hemorrhagic fever with renal syndrome (HFRS). Hantaviruses enhance vascular endothelial growth factor directed EC permeability resulting in the disassembly of inter-endothelial cell adherens junctions (AJs). Recent studies demonstrate that Slit2 binding to Robo1/Robo4 receptors on ECs has opposing effects on AJ disassembly and vascular fluid barrier functions. Here we demonstrate that Slit2 inhibits ANDV and HTNV induced permeability and AJ disassembly of pulmonary microvascular ECs (PMECs) by interactions with Robo4. In contrast, Slit2 had no effect on the permeability of ANDV infected human umbilical vein ECs (HUVECs). Analysis of Robo1/Robo4 expression determined that PMECs express Robo4, but not Robo1, while HUVECs expressed both Robo4 and Robo1 receptors. siRNA knock-down of Robo4 in PMECs prevented Slit2 inhibition of ANDV induced permeability demonstrating that Robo4 receptors determine PMEC responsiveness to Slit2. Collectively, this data demonstrates a selective role for Slit2/Robo4 responses within PMECs that inhibits ANDV induced permeability and AJ disassembly. These findings suggest Slit2s utility as a potential HPS therapeutic that stabilizes the pulmonary endothelium and antagonizes ANDV induced pulmonary edema.

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Hantaviruses predominantly infect human ECs and nonlytically cause two vascular leakage based diseases hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (Bustamante et al., 1997; Chang et al., 2007; Duchin et al., 1994; Enria et al., 1996; Galeno et al., 2002; Hallin et al., 1996; Koster et al., 2001; Levis et al., 1997; Lopez et al., 1996; Nolte et al., 1995; Padula et al., 1998; Schmaljohn and Hjelle, 1997; Zaki et al., 1995). Hantavirus infection alone does not cause EC permeability and instead hantaviruses alter EC responses that regulate fluid barrier properties of capillaries and lymphatic vessels (Gavrilovskaya et al., 2012b, 2008; Mackow and Gavrilovskaya, 2009). Currently there are no effective therapeutics for treating symptomatic HPS or HFRS patients (Jonsson et al., 2008).

Infection of ECs by pathogenic hantaviruses results in the hyper-phosphorylation of VEGFR2 and increased EC monolayer permeability in response to vascular endothelial growth factor (VEGF) (Gavrilovskaya et al., 2008; Gorbunova et al., 2010, 2011). HPS patients are acutely hypoxic with pulmonary edema fluid accumulating at up to a liter per hour (Koster and Mackow,

2012). VEGF is induced by hypoxic conditions and VEGF was first identified as a permeability factor that potently causes vascular leakage and edema (Dvorak, 2006). Hypoxia induced VEGF causes high altitude induced pulmonary edema, resulting from an auto-amplifying loop of VEGF induction that in turn increases the expression of the hypoxic sensor, HIF-1 α (Berger et al., 2005; Dehler et al., 2006; Hopkins et al., 2005; Kosmidou et al., 2008; Manalo et al., 2005).

Vascular integrity is critical to survival and capillary leakage is redundantly regulated by factors, receptors and signaling responses that act in concert to maintain fluid barriers (Gavard and Gutkind, 2006; Kosmidou et al., 2008; Nagy et al., 2012). A growing list of factors counter permeabilizing VEGF-VEGFR2 responses by antagonizing VE-cadherin disassembly and stabilizing AJs (Gavard et al., 2008; Koch et al., 2011; London et al., 2009; Robinson et al., 2004; Xu et al., 2007). Recently Slit2 has been shown to positively or negatively regulate VEGF directed permeability depending on its respective binding to Robo1 and Robo4 receptors (Acevedo et al., 2008; Koch et al., 2011; Sheldon et al., 2009). These findings suggest that Slit2 responses are determined by the tissue specific expression of Robo1/Robo4 receptors in ECs (Dickinson and Duncan, 2011; Sheldon et al., 2009).

Robo4 is expressed in pulmonary tissues suggesting the potential for Slit2 responses to inhibit hantavirus induced permeability

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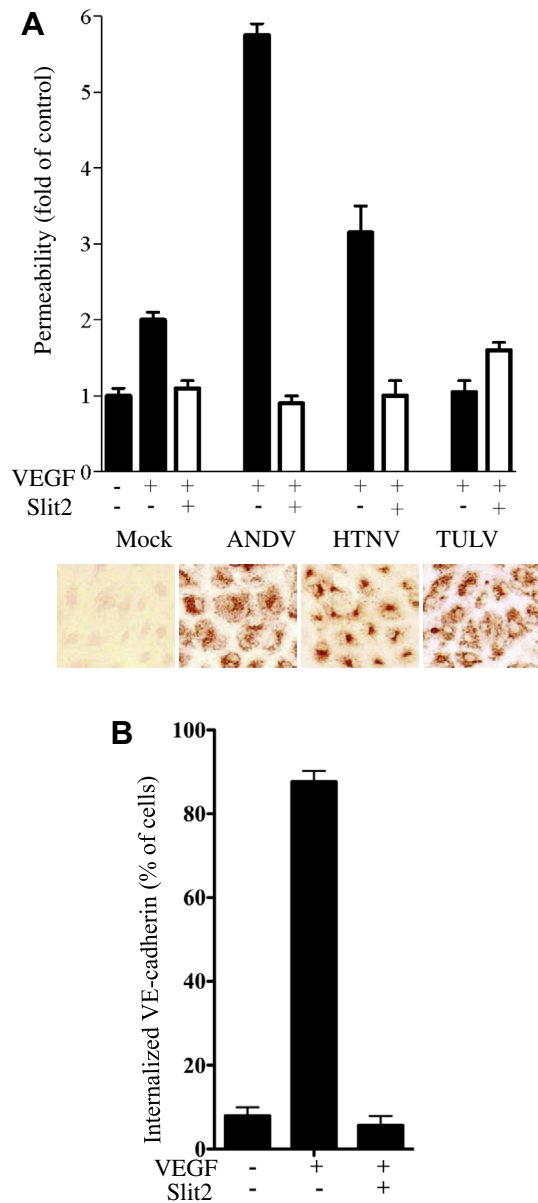


Fig. 1. Permeability and VE-cadherin internalization assays were performed 3 days post-infection as described (Gavrilovskaya et al., 2008). (A) Pulmonary microvascular ECs (PMECs/human microvascular ECs-lung-Cambrex) were mock, ANDV (CHI-7913), Hantaan virus (HTNV 76-118) or TULV (Tula/Moravia/MA 5302V/94) infected in BSL3 at an MOI of 0.5. In lower panel infected cells were immunoperoxidase stained for the hantavirus nucleocapsid protein (Geimonen et al., 2002). Three days p.i. monolayers were treated as indicated with VEGF (100 ng/ml) or Slit2 (N-terminal residues 1-1093 of human Slit2 homolog, Slit2-N; PeproTech, >98% pure) (10 nM) prior to assessing monolayer permeability to FITC-dextran (40,000; 0.5 mg/ml) as previously described. (B) PMECs were infected for 3 days and subsequently analysis of ANDV induced VE-cadherin internalization was performed as previously described on PMECs with or without Slit2 (Gavard and Gutkind, 2006; Gorbunova et al., 2010). Experiments were performed four times and error bars represent s.e.m.

in pulmonary settings (Jones et al., 2009; London and Li, 2011). The role of Slit-2 stabilizing fluid barrier functions of the vasculature (Jones et al., 2009) prompted us to determine if Slit2 inhibits hantavirus induced EC responses. We initially monitored the permeability of ANDV (HPS), HTNV (HFRS) and TULV (no associated human disease) infected PMECs in response to Slit2 addition (London and Li, 2011). PMECs were comparably infected by ANDV,

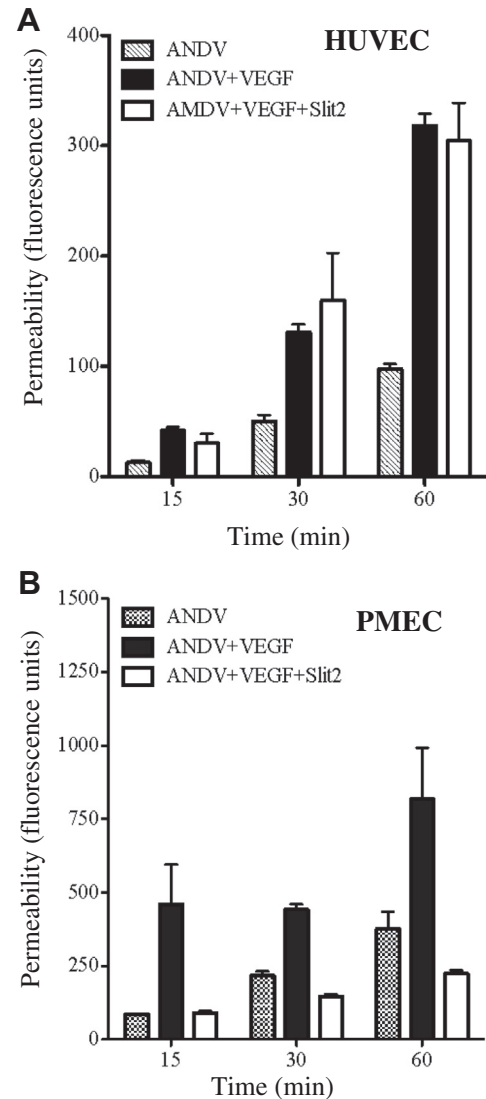


Fig. 2. PMECs or HUVECs were infected for three days at an MOI of 0.5 and subsequently assayed for the effect of Slit2 on ANDV permeability in (A) human umbilical vein ECs (HUVECs-Cambrex); or (B) PMECs 15, 30 and 60 min post-VEGF addition, was performed as in Fig. 1 and previously described (Gavrilovskaya et al., 2008).

HTNV and TULV as demonstrated by expression of the viral nucleocapsid protein within monolayers (Fig. 1A) (Gavrilovskaya et al., 1998). Pathogenic ANDV and HTNV enhanced PMEC permeability in response to VEGF (Gavrilovskaya et al., 2008; Raymond et al., 2005), however the addition of Slit2 to infected PMECs abrogated ANDV or HTNV monolayer permeability (Fig. 1A). To demonstrate that the effects were the result of changes in AJs we subsequently monitored the effect of Slit2 on VE-cadherin internalization (Gavard and Gutkind, 2006; Gorbunova et al., 2010). ANDV infection resulted in the internalization of VE-cadherin in 80–90% of PMECs. However, addition of Slit2 to PMECs reduced VE-cadherin internalization to background levels (Fig. 1B). These findings demonstrate that Slit2 regulates ANDV induced VE-cadherin disassembly within AJs of infected PMECs. In contrast, we found that ANDV induced HUVEC permeability responses were insensitive to Slit2 addition (Fig. 2A) and unique from Slit2 inhibition of permeability responses observed in PMECs at all time points after VEGF addition (Fig. 2B). This indicated that Slit2 regulates PMEC, but not HUVEC, permeability responses induced by ANDV, and suggests the importance of analyzing tissue specific EC responses.

Robo1 and Robo4 receptors elicit opposing permeability responses following Slit2 binding (Marlow et al., 2010). This suggested a role for discrete Robo1/Robo4 levels in the differential Slit-2 responsiveness of HUVECs and PMECs. We determined the relative mRNA levels of Robo1 and Robo4 in HUVEC and PMECs infected with ANDV, HTNV or TULV and found that HUVECs contained high levels of both Robo1 and Robo4 mRNAs (Fig. 3A), while Robo4 mRNA levels were >10-fold higher than Robo1 in hantavirus infected PMECs (Fig. 3B). These findings suggest that PMECs predominantly express Robo4 receptors which regulate EC permeability in response to Slit2. We probed the role of Robo4 expression in the Slit2 responsiveness of PMECs using an siRNA knockdown approach (Kaur et al., 2008). Compared to control siRNAs, Robo4 siRNAs reduced Robo4 mRNA levels >80% (Fig. 3C). Control siRNAs had no effect on the Slit2 responsiveness of ANDV infected PMECs (Fig. 3D). In contrast, PMECs transfected with Robo4-specific siRNAs failed to regulate ANDV directed permeability following Slit2 addition. These findings indicate that Slit2 inhibits ANDV-induced hyperpermeability of PMECs via a Robo4 dependent mechanism.

The ability of Slit2 to inhibit hantavirus permeability suggested that Slit2 effects might be additive to the effects of chemical inhibitors which block VE-cadherin internalization and PMEC perme-

ability responses through unique mechanisms. Fig. 4 compares Slit2 regulated permeability of ANDV infected PMECs to regulation by the Src inhibitor dasatinib and the S1P analog FTY720 (Gavrilovskaya et al., 2008; Gorbunova et al., 2011). Inhibitors were used individually or additively and assayed for their effect on ANDV induced monolayer induced permeability. Slit2 alone inhibited ANDV induced PMEC permeability similarly to dasatinib or FTY720 (Fig. 4). Slit2 addition in combination with dasatinib or FTY720 resulted in more effective inhibition of ANDV induced permeability responses than any compound applied individually (Fig. 4). These findings suggest that Slit2, alone or in conjunction with other compounds, may be useful in enhancing fluid barrier functions of the pulmonary endothelium during hantavirus infection.

Hypoxia induced VEGF reflects an apparent short term pulmonary repair mechanism that when sustained causes vascular dysfunction and edema (Berger et al., 2005; Stenmark et al., 2006; Tang et al., 2004). VEGF is an EC specific growth and permeability factor that acts on cells within 0.5 mm from its release and both directs EC migration and localized permeability of the endothelium (Dvorak, 2006; Dvorak et al., 1991). HPS patients are acutely hypoxic, have elevated levels of VEGF in their pulmonary edema fluid

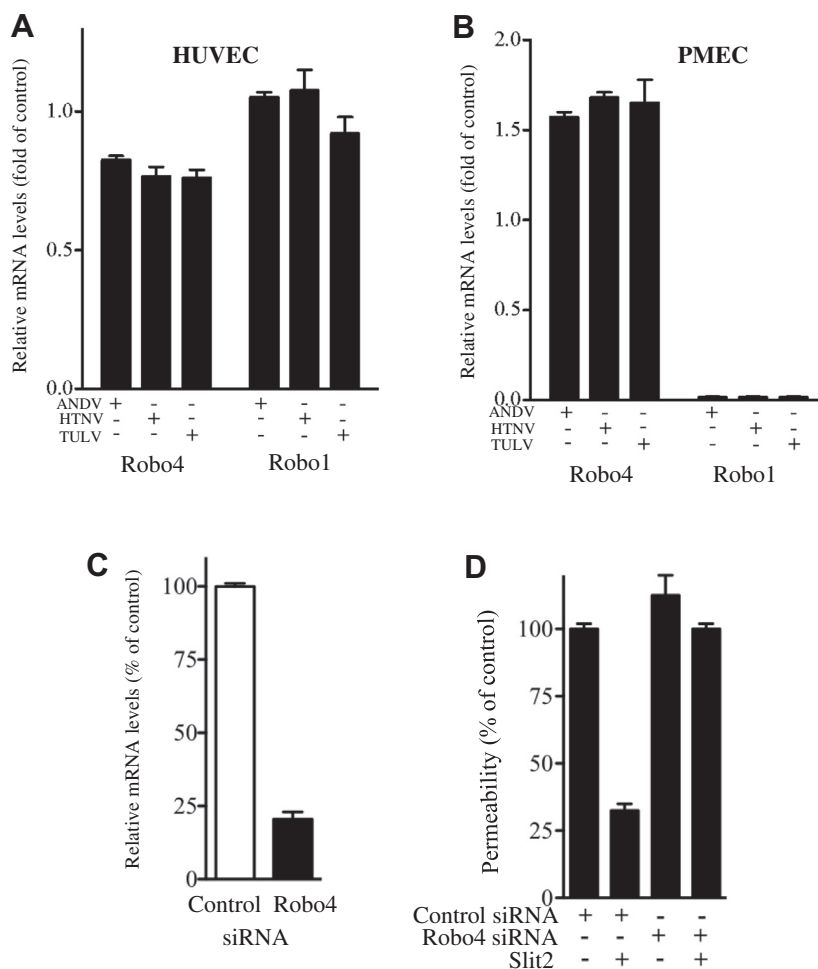


Fig. 3. Role of Robo1/4 in Slit2 regulation of PMECs. HUVECs (Pepini et al., 2010) (3A) or PMECs (3B) were infected as above and analyzed by qRT-PCR for Robo1 and Robo4 mRNAs relative to GAPDH mRNA levels (Pepini et al., 2010). (3C) PMECs were transfected with negative-control (si-NEG2) or Robo4-specific siRNA and assayed by qRT-PCR for Robo4 mRNA levels as in 3B (Pepini et al., 2010). (3D) PMECs were ANDV infected as in Fig 1, Robo4 or control siRNA transfected 1 day post-infection, as in 3C, and assayed for PMEC permeability in response to VEGF as in Fig 1.

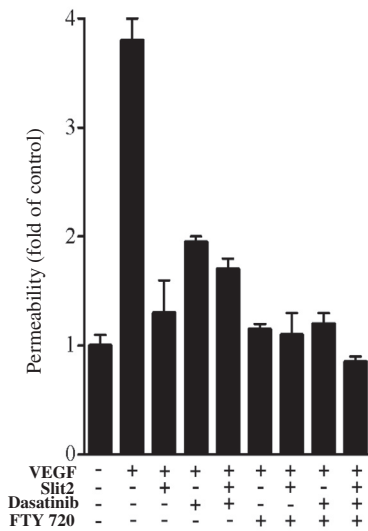


Fig. 4. PMECs were ANDV infected and assayed for changes in permeability as in Fig. 1 in the presence or absence of Slit2 (effective range 5–50 nM), dasatinib or FTY720 (Gavrilovskaya et al., 2008; Gorbunova et al., 2010, 2011).

and patient hyper-oxygenation has contributed to a substantial reduction in HPS mortality rates (~85% to ~35%) (Gavrilovskaya et al., 2012a; Koster and Mackow, 2012; Zaki et al., 1995). HPS patients often present in respiratory distress weeks after initial infection and rapidly progress to a critical disease period in which they accumulate pulmonary edema fluid at up to 1 L per hour (Koster and Mackow, 2012). Both increased capillary permeability and decreased fluid clearance may contribute to HPS and hantaviruses infect the EC lining of vascular and lymphatic vessels (Koster and Mackow, 2012).

In vitro, pathogenic hantavirus infection of microvascular and lymphatic ECs enhances VEGF directed VEGFR2 phosphorylation, EC permeability and AJ disassembly (Gavrilovskaya et al., 2010, 2012b, 2008; Gorbunova et al., 2010, 2011; Macneil et al., 2011; Shrivastava-Ranjan et al., 2010). These findings and the acute state of symptomatic hantavirus patients suggest targeting the endothelium, rather than virus, in order to stabilize and restore normal vascular barrier and clearance functions (Gavrilovskaya et al., 2012b, 2008; Gorbunova et al., 2011). Compounds that directly target VEGFR2 induced changes in AJs (i.e. pazopanib/dasatinib) as well as growth factors and lipid mediators (i.e. Ang-1, S1P/FTY720), that act through unique EC receptors, have the potential to restrict hantavirus directed EC permeability (Gavrilovskaya et al., 2008; Gorbunova et al., 2011). Studies presented here demonstrate that Slit2, a naturally secreted protein, restricts ANDV induced permeability of pulmonary ECs by binding Robo4 receptors. Our findings suggest that Slit2, alone or in conjunction with additional vascular barrier enhancers, may serve to prevent ANDV induced vascular permeability by stabilizing VE-cadherin within inter-EC junctions (Gavrilovskaya et al., 2008). These existing compounds and additional receptor and pathway specific inhibitors may provide therapeutic approaches for reducing the severity of hantavirus diseases at late times after infection when antivirals are ineffective (Jonsson et al., 2008).

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