Early Activation of Primary Brain Microvascular Endothelial Cells by Nipah Virus Glycoprotein-Containing Particles

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Nipah virus (NiV) is a highly pathogenic paramyxovirus that causes pronounced infection of brain endothelia and central nervous system (CNS) inflammation. Using primary porcine brain microvascular endothelial cells, we showed that upregulation of E-selectin precedes cytokine induction and is induced not only by infectious NiV but also by NiV-glycoprotein-containing virus-like particles. This demonstrates that very early events in NiV brain endothelial infection do not depend on NiV replication but can be triggered by the NiV glycoproteins alone.

Nipah virus (NiV) is a zoonotic paramyxovirus with a broad host range. Bats of the genus *Pteropus* represent the natural reservoir that can sporadically transmit the virus to other mammalian species. Southeast Asia is an area of Nipah virus disease endemicity, and disease outbreaks in pigs and humans have been reported in Malaysia, Singapore, India, and Bangladesh (1, 2). Clinical NiV infections are generally characterized by respiratory and encephalitic symptoms. Histopathological analyses revealed pronounced endothelial tropism. Brain microvascular endothelial cells represent major target cells of *in vivo* infections and are primarily responsible for NiV-induced central nervous system (CNS) inflammation (3, 4). Previous studies had shown that NiV

infection of endothelia leads to upregulation of cytokines such as beta interferon (IFN- β), interferon-inducible protein-10 (IP-10),

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FIG 1 NiV infection of PBMEC. (A) PBMEC were grown to confluence for 5 days, fixed, and permeabilized using 4% paraformaldehyde (PFA) and 0.1% Triton X-100. ZO-1 and VE-cadherin were stained using specific mouse monoclonal antibodies (Life Technologies, Santa Cruz Biotechnology) and rhodamine- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Jackson Immuno Research), respectively. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). Bars, 20 μ m. The insets represent expansions of the boxed regions. (B to D) PBMEC were infected with NiV stock virus pelleted from Vero cell culture supernatants at a multiplicity of infection (MOI) of 2. At 4 h and 17 h p.i., total RNA was extracted and reverse transcribed using random hexamer primers and cDNAs were analyzed by quantitative real-time PCR (SYBR green; Applied Biosystems) using specific primers for NiV-N (B), for E-selectin (C), or for IFN- β and IP-10 (D). The values corresponding to fold change compared to the results of mock treatment are shown. Bars indicate the standard deviations. Statistical significance: *, $P < 0.05$; **, $P < 0.01$.

TABLE 1 qPCR primers used in this study

Primer	Sequence
NiV-N for	ATCAATCGTGGTTATCTTGA
NiV-N rev	CAGCCAGTTCTGCAACTTGATC
E-selectin for	TGTGCCTTCAGCTGTGAGG
E-selectin rev	AGCCAGAGGACAAATGATGC
IFN- β for	GCTAACAAGTGCATCCTCCAAA
IFN- β rev	AAGCACATCATAGCTCATGGAAG
IP-10 for	CCATGGATTGCAGTCACCAA
IP-10 rev	GCTCGGGATGATGAACCATCT
Tubulin for	CTGAACCGCCTTATTAGCCAAA
Tubulin rev	CGTTCAGGGCCCCATCA

and interleukin-8 (IL-8), accompanied by a disturbance of the blood-brain barrier (5–7). The aim of this work was to determine the requirements for early events in endothelial cell activation. For our studies, primary brain microvascular endothelial cells (PBMEC) were freshly isolated from pig brains as described previously (8, 9). After endothelial integrity was controlled by immunostaining of endothelial adherens and tight junction marker proteins, namely, vascular endothelial (VE)-cadherin and ZO-1 (Fig. 1A), PBMEC were infected with a cytokine-free NiV stock virus at a multiplicity of infection (MOI) of 2. At 4 h and 17 h postinfection (p.i.), total RNA was isolated and analyzed by qPCR. To control productive virus infection (Fig. 1B), the amount of viral RNA in infected cell lysates was quantified using NiV nucleoprotein-

specific primers (Table 1). E-selectin-specific quantitative PCR (qPCR) revealed a pronounced upregulation of this early endothelial adhesion marker at 4 h p.i. (Fig. 1C). At 17 h p.i., E-selectin mRNA levels had declined, likely due to the lack of continuous stimulation (10). As IFN- β and IP-10 are described as hallmark cytokines in NiV infections (5–7), we hypothesized that these might be linked to or even responsible for E-selectin induction. However, when we analyzed IFN- β and IP-10 expression in infected PBMEC, interestingly, we found both cytokines to be induced at 17 h p.i. but not at 4 h p.i. (Fig. 1D). Thus, E-selectin upregulation is clearly independent of and precedes cytokine induction.

To determine if virus replication or viral RNA is needed for early endothelial activation, we generated virus-like particles (VLPs) (11) containing all three envelope-associated NiV proteins (FGM-VLPs), containing the receptor-binding G glycoprotein and the matrix protein M (GM-VLPs), or containing only M protein and no glycoproteins (M-VLPs). Western blot analysis of purified VLPs confirmed the expected protein composition (Fig. 2A). To functionally test FGM-VLPs, we performed an overlay fusion assay. As shown in Fig. 2B, only FGM-VLPs but not F-deficient GM- or M-VLPs were able to cause cell fusion from without (11, 12). To further characterize the different VLPs, we tested their influence on transendothelial permeability in PBMEC cultivated on Transwell filters. In agreement with our earlier observa-

FIG 2 Characterization of NiV-glycoprotein-containing VLPs. VLPs were prepared basically as described by Walpita et al. (11). Briefly, 5.4×10^7 subconfluent 293 cells were transfected with different combinations of NiV-F-, G-, and M-encoding plasmids (13) using polyethylenimine (PEI) transfection reagent. Forty-eight hours later, supernatants were cleared and pelleted through a 20% sucrose cushion. VLPs were then suspended in a total volume of 100 μ l. (A) Five percent (5 μ l) of each of the FGM-, GM-, and M-VLP samples was separated by SDS-PAGE under reducing conditions and blotted to nitrocellulose. Viral proteins were detected by the use of F-, G-, and M-specific polyclonal rabbit antibodies (ImmunoGlobe) and IRDye 800-conjugated secondary antibody (Life Technologies) and visualized by using an Odyssey image analyzer (Li-COR). (B) Ninety-five percent (95 μ l) of FGM-VLP, GM-VLP, or M-VLP preparations was added to confluent Vero cells. After 24 h, cells were stained with Giemsa staining solution. Cells in syncytia were visualized using a phase-contrast microscope (Peqlab) (magnification, $\times 200$). (C) PBMEC were cultured for 5 days on polycarbonate Transwell filters (Greiner). Confluent cell monolayers were then incubated for 6 h with medium (mock) or with 95 μ l (95%) of FGM-, GM-, or M-VLPs prepared from 5.4×10^7 transfected 293 cells. Horseradish peroxidase (HRP; 5 μ g/ml) was added to the apical filter chamber. After 1.5 h, apical-to-basolateral HRP passage was quantified by measuring the HRP activity in the basal medium, and data are given as means of the results of 3 replicates normalized to the HRP concentration in the basal medium of a filter without cells (set = 1). rel., relative. Statistical significance: *, $P < 0.05$.

FIG 3 PBMEC activation by VLPs. Confluent PBMEC were incubated for 60 min with 95% (95 μ l) FGM-, GM-, or M-VLPs prepared as described in the legend to Fig. 2. After 2 h and 4 h, total RNA was extracted and qPCR was performed as described in the legend to Fig. 1. The values corresponding to fold change compared to the results seen with M-VLPs are shown. Statistical significance: **, $P < 0.01$.

tion for NiV-infected polarized cell monolayers (8, 13), FGM-VLPs induced an increased apical-to-basal flowthrough of horseradish peroxidase (Fig. 2C). This was not seen for GM- and M-VLPs, thereby supporting our model that fusion events are largely responsible for the changes in transendothelial permeability (8).

To test the ability of VLPs to activate PBMEC, we added FGM-, GM-, or M-VLP preparations for either 2 h or 4 h to confluent endothelial monolayers and measured upregulation of E-selectin, IFN- β , and IP-10 (Fig. 3). qPCR data were normalized to M-VLP-treated PBMEC data. As shown in Fig. 3A, we found a more prominent E-selectin upregulation 2 h after addition of FGM-VLPs than after 4 h. GM-VLPs also induced some E-selectin expression but did so to clearly lower levels (Fig. 3A). It therefore can be concluded that adhesion molecule upregulation is a very rapidly triggered event that needs neither NiV RNA nor virus replication but is directly caused by binding of particles containing the NiV G protein. Activation is further enhanced if particles also contain the F protein and therefore can cause fusion from without. As seen in the infection studies (Fig. 1), the activation is independent from cytokine production. Neither IFN- β nor IP-10 was found to be induced after 2 h of FGM- or GM-VLP incubation (Fig. 3B), when E-selectin was already upregulated.

Our study revealed an early and significant induction of E-selectin in primary brain endothelial cells after either NiV infection or incubation with VLPs. IFN- β and IP-10 upregulation was seen much later in infection and was undetectable (IFN- β) or less prominent (IP-10) in the absence of a virus infection. This suggests that these key cytokines found in *in vivo* infections (6, 7) were primarily induced by viral RNA and/or virus replication, likely by RIG-I-dependent pathways (14). In contrast, E-selectin expression, the starting point for recruiting leukocytes to the endothelium, appears to be directly induced after binding and fusion of viruses or virus-like particles. This shows for the first time that particles containing NiV-G and NiV-F proteins can upregulate the adhesion molecules known to be implicated in early steps of inflammation events in microvascular brain endothelial cells.

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