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Equine herpesvirus type 1 modulates inflammatory host immune response genes in equine endothelial cells

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ABSTRACT

Equine herpesvirus myeloencephalopathy (EHM), a disease caused by equine herpesvirus type 1 (EHV-1), is characterized by severe inflammation, thrombosis, and hypoxia in central nervous system (CNS) endothelial cells, which can result in a spectrum of clinical signs including urinary incontinence, ataxia, and paralysis. Strains of EHV-1 that contain a single point mutation within the viral DNA polymerase (nucleotide $A_{2254} > G_{2254}$: amino acid $N_{752} \rightarrow D_{752}$) are isolated from EHM afflicted horses at higher frequencies than EHV-1 strains that do not harbor this mutation. Due to the correlation between the DNA Pol mutation and EHM disease, EHV-1 strains that contain the mutation have been designated as neurologic. In this study, we measured virus replication, cell to cell spread efficacy, and host inflammatory responses in equine endothelial cells infected` with 12 different strains of EHV-1. Two strains, T953 (Ohio 2003) (neurologic) and Kentucky A (KyA) (non-neurologic), have well described disease phenotypes while the remaining strains used in this study are classified as neurologic or non-neurologic based solely on the presence or absence of the DNA pol mutation, respectively. Results show that the neurologic strains do not replicate better or spread more efficiently in endothelial cells. Also, the majority of the host inflammatory genes were modulated similarly regardless of EHV-1 genotype. Analyses of host gene expression showed that a subset of pro-inflammatory cytokines, including the CXCR3 ligands CXCL9, CXCL10, and CXCL11, as well as CCL5, IL-6 and TNF-α w ere consistently up-regulated in endothelial cells infected with each EHV-1 strain. The identification of specific pro-inflammatory cytokines in endothelial cells that are modulated by EHV-1 provides further insight into the factors that contribute to the immunopathology observed after infection and may also reveal new targets for disease intervention.

1. Introduction

Equine herpesvirus type 1 (EHV-1) is a major pathogen of equines and causes considerable morbidity and mortality in horses (Allen and Bryans, 1986). Horses develop a respiratory infection after exposure to EHV-1 and exhibit clinical signs, which include fever, malaise, mucopurulent discharge, and inappetance. Most horses recover without complications within 1–2 weeks post-infection, but more serious sequelae can develop, including abortions and the severe neurological disease, equine herpesvirus myeloencephalopathy (EHM) (Pusterla and Hussey, 2014).

EHM has been shown to be a disease primarily caused by severe inflammation within the central nervous system (CNS) (Pusterla and Hussey, 2014). Infection of CNS endothelial cells consequent to a cell-associated viremia results in thrombosis, ischemia, and hypoxia which damage the adjacent neurons (Edington et al., 1986; Kydd et al., 2012; Pusterla and Hussey, 2014; Smith et al., 2002). The importance of endothelial cells to the pathophysiology of EHV-1 mediated neurologic disease was described initially by Edington et al. who showed that EHV-1 infection within CNS endothelial cells is similar to that observed in EHV-1 infected endothelial cells in the pregnant uterus (Edington et al., 1986). Within both anatomical sites, severe thrombosis, hemorrhaging, and necrosis of endothelial cells was observed. The degree of infection and consequent damage to the endothelium was further shown to vary depending upon the infecting strain of EHV-1 (Patel et al., 1982) and studies performed in horses revealed a positive correlation between disease outcome and efficiency of endothelial cell infection (Smith et al., 1993). Neurologic EHV-1 strains such as Ab4, which are highly virulent, readily infected endothelial cells while less virulent strains such as the non-neurologic V592 were less able to infect these cells (Smith et al., 2000).

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Studies have shown that strains of EHV-1 that contain a point mutation ($A_{2254} > G_{2254}$) within the DNA polymerase (DNA Pol: $N_{752} \rightarrow D_{752}$) are more likely to cause EHM versus other strains (Goodman et al., 2007; Nugent et al., 2006; Perkins et al., 2009; Smith et al., 2010). The mutation consists of a non-synonymous A-to-G substitution at nucleotide 2254 ($A \rightarrow G_{2254}$) in the DNA Pol and results in a change from an asparagine to aspartic acid residue at amino acid 752 ($N_{752} \rightarrow D_{752}$). Data from various groups have shown that EHV-1 strains with this DNA Pol mutation are isolated at a higher frequency from horses afflicted with EHM compared to EHV-1 strains that do not contain this mutation (Allen, 2007; Perkins et al., 2009; Pusterla and Hussey, 2014). Thus, EHV-1 strains that harbor the DNA pol mutation ($A_{2254} > G_{2254}$) are now commonly classified as neurologic while those that do not harbor this mutation are classified as non-neurologic.

While the DNA pol mutation is correlated with neurovirulence, it is not the sole factor that influences whether an infected horse will develop EHM. The currently available data support the hypothesis that EHM is multifactorial as 16–24% of EHM cases are caused by an EHV-1 strain that does not contain the DNA pol mutation (Perkins et al., 2009). Therefore, researchers and clinicians should be cautious when ascribing neurologic potential of EHV-1 based solely on the presence of the DNA Pol mutation.

We hypothesize that the pattern and degree of host pro-inflammatory responses elicited after endothelial cell infection will have prognostic value for the classification of EHV-1 strains as neurologic or non-neurologic. Previous research identified a number of pro-inflammatory proteins that contribute to EHV-1 induced immunopathology in equines and/or tissue/animal models. Wimer et al. showed that CCL5 is up-regulated in PBMC after infection with multiple EHV-1 strains (Wimer et al., 2011). In a separate study examining the inflammatory response to EHV-1, Soboll Hussey et al. showed that TNF-α and other pro-inflammatory chemokines are significantly increased following infection of equine epithelial and PBMC with the neurologic EHV-1 strain Ab4 (Soboll Hussey et al., 2014). Also, in a murine model of EHV-1 pathogenesis, we previously showed that the production of the pro-inflammatory mediators MIP-1α, MIP-2β, and TNF-α are highly correlated with severe lung immunopathology (Frampton et al., 2002).

Since the degree of inflammation within the endothelium of EHV-1 infected horses is a key determinant of EHM disease, we tested the hypothesis that EHV-1 strains designated as neurologic (DNA Pol $A_{2254} > G_{2254}$) will elicit a more severe pro-inflammatory response in endothelial cells compared to the non-neurologic strains. To test this hypothesis, we first infected equine endothelial cells with two EHV-1 strains with a well-defined disease phenotype, the neurovirulent T953 strain (Henninger et al., 2007) and the highly attenuated Kentucky A (KyA) strain (Matsumura et al., 1996), and then measured and compared the expression levels of host immune response genes after infection. After assessing the host immune response to these two strains, we next examined the immune response to a series of EHV-1 strains that are classified as neurologic or non-neurologic based solely on whether they contain the DNA Pol mutation ($A_{2254} > G_{2254}$) or not. In addition to evaluating the host immune response to the EHV-1 strains, we also examined whether neurologic strains were better able to replicate and/or spread from cell-to-cell compared to non-neurologic strains in equine endothelial cells.

2. Materials and methods

2.1. Cells and viruses

Rabbit kidney (RK-13) cells were provided by Dennis O'Callaghan (Louisiana State University Health Sciences Center, Shreveport, LA) and the equine cardiac artery endothelial (EE) cell line (Hedges et al., 2001) was provided by Udeni Balasuriya (University of Kentucky Maxwell H. Gluck Equine Research Center, Lexington, KY). All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% of 10,000 U/mL penicillin and 10,000 μg/mL streptomycin in normal saline (P/S) (JR Scientific, Inc., Woodland, CA). Cells were maintained at 37 °C, 5% CO_2.

EHV-1 strains used in this study include: T953 (Ohio 2003) (Henninger et al., 2007), KyA (Turtinen et al., 1981), T313, T954, T964, T967, T970, T61, T75, T220, T493, T547 (Smith et al., 2010). EHV-1T strains that harbor the DNA Pol mutation ($A_{2254} > G_{2254}$) were classified as neurologic and those that do not contain the mutation were classified as non-neurologic. T953 was kindly provided by Gillian Perkins (Cornell University, College of Veterinary Medicine), and T313, T954, T964, T967, T970 T61, T75, T220, T493, T547, were generously provided by Udeni Balasuriya.

2.2. EHV-1 replication assays

Confluent monolayers of EE cells (8×10^5 cells/well in a 24-well plate) were infected with each EHV-1 strain at an MOI of 1 and incubated at 37 °C, 5% CO_2 for 24 h. For each strain, a total of 3 biological replicates were performed. Twenty-four hours post-infection (p.i), the cells and supernatants were collected, placed into 1.7 mL microcentrifuge tubes and centrifuged at 13,000 RPM for 10 min. Supernatants was transferred to new 1.7 mL micro-centrifuge tubes and stored on ice. The remaining cell pellets were re-suspended in 50 μL of the supernatant, and the samples were subjected to three freeze-thaw cycles between −80 °C and 37 °C to release cell-associated virus. After three freeze-thaw cycles, the disrupted cell pellets were added back to their respective supernatants. Virus was titered on RK-13 cells. The data represent the mean titers (pfu/mL)+/− standard error of the three biological replicates. The means of the neurologic and non-neurologic strains were calculated and the variation between these groups was evaluated using a Student's two tailed *t*-test.

2.3. Plaque phenotype of EHV-1 strains in EE cells

Confluent monolayers of EE cells in 6-well plates were infected with approximately 100 plaque forming units (pfu) of each EHV-1 strain at 37 °C, 5% CO_2 for 2 h. After 2 h, cells were overlaid with 5 mL of semi-solid complete DMEM containing 1% methylcellulose, and incubated for 72 h. After the 72 h incubation, semi-solid media was aspirated off and the cells were stained with 1% crystal violet for 60 min. Stained cells were examined under a Motic inverted light microscope (Motic, Causeway Bay, Hong Kong). Plaques (at least 25 plaques/EHV-1 strain) were imaged using a STEMI SV6 Zeizz inverted microscope using 0.8× magnification accompanied by Spot Advance computer software and images were captured using an RT Color Spot Diagnostic Instruments, Inc. camera. Plaque areas were measured using Image Pro Plus software and mean plaque sizes (mm^2) for each strain were graphed. Data represent mean plaque size (mm^2) ($n \geq 25$ plaques/EHV-1 strain) and standard error. The mean plaque sizes of the neurologic and non-neurologic strains were calculated and the variation between these groups was evaluated using a Student's two-tailed *t*-test.

2.4. Assessment of host immune response gene expression in EE cells infected with EHV-1

EE cells (1.2×10^6 cells per well) were seeded in a 12 well plate. EHV-1 strains were added to the cells at an MOI of 3 for 10 h. Ten hours p.i., RNA was isolated using the RNeasy Plus Mini Kit (Qiagen,