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Impact of a cytomegalovirus kinase inhibitor on infection and neuronal progenitor cell differentiation

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ABSTRACT

Human cytomegalovirus (HCMV) is the leading cause of congenital infections. Symptomatic newborns present with a range of sequelae including disorders of the CNS such as visual impairment, microcephaly, mental retardation and hearing loss. HCMV congenital infection causes gross changes in brain morphology and disturbances in glial and neuronal distribution, number and migration. In these studies, we have evaluated the effectiveness of the antiviral maribavir in inhibiting HCMV infections of ES cell-derived neuronal progenitor cells (NPC). We used EZ-spheres generated from H9 ES cells which are pre-rosette NPCs that retain long-term potential to differentiate into diverse central and peripheral neural lineages following directed differentiation. Our results demonstrate that the maribavir disrupts HCMV replication and viral yield in undifferentiated EZ-sphere-derived NPCs. In addition, we observed that maribavir limits HCMV replication and reduces the percentage of infected cells during differentiation of NPCs. Finally, early steps in differentiation are maintained during infection by treating with maribavir, likely an indirect effect resulting from decreased viral spread. Future studies of NPC proliferation and differentiation during infection treated with maribavir could provide the impetus for studying maribavir as an antiviral agent for congenital HCMV disease.

1. Introduction

Human cytomegalovirus (HCMV) is the leading cause of congenital infections in developed countries (Demmler-Harrison, 2009) and likely around the world (Lanzieri et al., 2014). Congenital HCMV disease is the last of the major congenital infections that remains as a major health burden. While other congenital infections have been largely controlled by immunization and prevention, there is currently no vaccination or treatment to combat congenital HCMV. Furthermore, the mechanisms of pathogenesis within the developing brain are not yet fully understood. The majority of infected infants exhibit no clinical symptoms at birth but may develop hearing loss in the first few years of life (Yinon et al., 2010; Cannon et al., 2013). Symptomatic newborns present with a range of sequelae including jaundice, petechiae, hepatosplenomegaly, febrile syndrome, thrombocytopenia, anemia, and disorders of the CNS such as visual impairment, microcephaly, mental retardation and hearing loss (Cannon et al., 2013). The mechanisms leading to disease are poorly understood but involve neuronal damage (Tsutsui, 2009; Kawasaki and Tsutsui, 2003; Teissier et al., 2014) and impaired placental development (Pereira and Maidji, 2008; Pereira et al., 2014; Tabata et al., 2015). Studies also suggest that the late onset of hearing loss involves persistent inflammation (Tabata et al., 2015; Davis et al., 1981).

In human tissue and animal models, congenital HCMV infection causes gross changes in brain morphology and disturbances in glial and neuronal distribution, number and migration (Yinon et al., 2010; Pan et al., 2013). Cells in the subventricular zone (SVZ) were found to be especially susceptible to HCMV infection (Tsutsui, 2009; Kawasaki and Tsutsui, 2003; Teissier et al., 2014). The SVZ is made up primarily of neural progenitor cells (NPCs) (Borrell and Calegari, 2014). NPCs derived from fetal tissue, induced pluripotent stem (iPS) cells, or embryonic stem (ES) cells are permissive to HCMV infection, as are glial and neuronal cells (Belzile et al., 2014; Luo et al., 2008; D'Aiuto et al., 2012). On the other hand, embryonic stem cells and primitive pre-rosette neural stem cells show low

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susceptibility to lytic HCMV infection, though the virus can enter and maintain its genome in these cells (Belzile et al., 2014; Penkert and Kalejta, 2013). HCMV infection of NPCs results in cytopathic effect, decreased proliferation, loss of multipotency markers, and cell death (Luo et al., 2008; D'Aiuto et al., 2012; Luo et al., 2010; Odeberg et al., 2006; Cheeran et al., 2005).

There are currently no approved therapies for use in congenital HCMV infection (McGregor et al., 2013; Adler and Nigro, 2013). The anti-HCMV agents are not approved for use during pregnancy due to concerns about teratogenesis, impaired fertility and bone marrow suppression in fetuses from animal studies (Adler and Nigro, 2013). Hyper immunoglobulin has been used with some success in small studies (Adler and Nigro, 2013). In addition, ganciclovir and valganciclovir have been used to treat infants following congenital HCMV infection with positive results, though toxicity remains a concern (Adler and Nigro, 2013). Identifying treatments that could be used safely in newborns and during pregnancy is a primary focus of HCMV research. The anti-HCMV compound maribavir (MBV) specifically inhibits the viral kinase, pUL97 (Biron et al., 2002; Trofe et al., 2008). Though MBV has been screened for inhibition against other kinases, off-target effects have yet to be identified (Biron, 2011; Nguyen et al., 2002). Toxicity in humans is low and includes manageable symptoms such as taste disturbances, headache and nausea (Biron et al., 2002). MBV was not genotoxic or teratogenic in animal studies (Biron, 2011; Nguyen et al., 2002). In addition, MBV demonstrates high bioavailability, can be taken orally, and crosses the blood–brain barrier (Biron et al., 2002).

We have evaluated the effectiveness of MBV in inhibiting HCMV infections of ES cell-derived NPC. We found that MBV inhibited viral protein expression and viral yield while maintaining expression of a subset of NPC markers in both undifferentiated and differentiated conditions. This study provides evidence that MBV may be a valuable anti-HCMV agent for treatment of congenital HCMV infection.

2. Material and methods

2.1. Biological reagents

Viral stocks were prepared by co-transfecting BAC DNA with the pCGN-pUL82HA expression vector into primary human foreskin fibroblasts (HFF). The wild-type TB40/E Bac4 (Sinzger et al., 2008) or TB40/E Bac4 containing the mCherry gene expressed from an SV40 promoter, generously provided by Dr. Eain Murphy (Cleveland Clinic), were used. The pCGN-UL82HA plasmid was generously provided by Dr. Robert Kalejta (University of Wisconsin–Madison) (Kuny et al., 2010). To concentrate viral stocks, culture media and cleared cell lysate was pelleted through a sorbitol cushion (20% D-sorbitol, 50 mM Tris–HCl pH 7.2, 1 mM $MgCl_2$) at 55,000 × g for 1 h. Virus was reconstituted in DMEM. Titers were defined as infectious units (IU) per ml and determined by using serial dilutions of viral stocks to infect HFFs in 12-well dishes, fixing cells at 48 h post infection (hpi), staining with an antibody against IE1, and counting IE1-positive cells per well.

Neural progenitor cells (NPCs) were derived from the H9 human embryonic stem (ES) cell line (NIH Stem Cell Registry 0062) as described by Ebert et al (Ebert et al., 2013). Briefly, ES cells were cultured in feeder free conditions on Matrigel in Nutrastem medium (Stemgent). Intact colonies were removed following dispase treatment and cultured as floating aggregates in NPC media, consisting of Stemline media (Sigma–Aldrich) supplemented with 100 ng/ml basic fibroblast growth factor (FGF-2, EMD Millipore), 100 ng/ml epidermal growth factor (EGF, EMD Millipore), and 5 μg/ml heparin (Sigma–Aldrich). The cells were cultured in ultra-low attachment flasks and were passaged weekly using a chopping technique for 11–16 passages (Ebert et al., 2008; Svendsen et al., 1998). To promote differentiation, 24 h before infection, cells were dissociated and plated in 24 well plates at 50,000 cells/well in DMEM/F12 supplemented with 2% B27 (ThermoFisher Scientific). Cells were infected at a multiplicity of infection of 1 IU per cell in NPC media containing 1% pen/strep or in DMEM/F12 supplemented with 2% B27 containing 1% pen/strep and exposed to the viral inoculum for 2 h, unless otherwise noted. Cells were analyzed 2–4 weeks post-infection. Compounds were added to the culture media at the start of infection and reapplied after removing the inoculum. Cells were treated with maribavir (provided by Shire) ranging from 1.25 to 40 μM to identify toxicity. Based on toxicity, all other experiments were carried out using 1 μM maribavir, or DMSO as a vehicle control (Sigma–Aldrich).

Antibodies used for Western blot (WB) or immunofluorescence analysis (IF) are listed below: Mouse anti-GAPDH clone 0411 (WB) (Santa Cruz Biotechnology); mouse anti-IE1 clone 1B12 (WB, IF) and mouse anti-pUL83 clone 8F5 (WB) were generously provided by Dr. T. Shenk (Princeton University); mouse anti-pUL44 (WB) (Virusys); mouse anti-nestin (IF) (EMD Millipore); mouse anti-Tuj1 (IF) (Sigma–Aldrich); rabbit anti-GFAP (IF) (Dako); goat anti-mouse IgG HRP and donkey anti-rabbit IgG HRP (WB) (Jackson ImmunoResearch); donkey anti-mouse IgG (H + L) Alexa Fluor 488; goat anti-rabbit IgG Alexa Fluor 568, and donkey anti-mouse IgG (H + L) Alexa Fluor 568 (IF) (ThermoFisher Scientific); and mouse Zenon AF488 (IF) (ThermoFisher Scientific).

2.2. Analysis of protein

Western blot analysis of steady-state protein levels were performed on cells lysed by sonication in lysis buffer (50 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.5% NP-40). Protein from lysates were resolved by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by semi-dry transfer to PROTRAN nitrocellulose membrane (GE Healthcare Life Sciences). Blocking was completed in 5% milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T). Membranes were incubated in primary antibody followed by a secondary antibody conjugated to HRP, both diluted in 5% milk in PBS-T. Antibodies were detected with ECL (GE Healthcare Life Sciences).

For immunofluorescence analysis, cells were plated approximately 50,000 cells per well in 24-well dishes containing glass cover slips. Immunofluorescence was initiated by fixing and permeabilizing cells in 100% methanol at −20 °C for 1 h or fixed in 4% paraformaldehyde for 15 min at room temperature followed by permeabilizing with 0.1% Triton-X for 15 min at room temperature. Blocking was performed with 3% BSA PBS-T for 30 min. Primary antibody was diluted in 3% BSA PBS-T was added for 1.5 h at RT then washed off three times with PBS-T. Secondary antibody conjugated to Alexa Fluor was diluted in 3% BSA PBS-T and incubated on cells for 30 min. Cover slips were put onto glass slides in ProLong Gold antifade reagent (ThermoFisher Scientific). Morphology was quantified at 200X and images were obtained at 400X on a Nikon Eclipse TS100 inverted microscope.

2.3. Cell toxicity assay

NPCs were treated with increasing concentrations of MBV from 1 to 40 μM. Cells were lifted from plates using trypsin, pelleted by centrifugation, and resuspended in a 1:20 dilution of crystal violet (EMD Millipore). Toxicity was determined by counting 100 cells from two biological replicates and scored as crystal violet positive (toxic) or negative. Cells were counted on a Nikon Eclipse TS100 inverted with the 10X objective lens.