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Pseudovirus mimics cell entry and trafficking of the human polyomavirus JCPyV

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ABSTRACT

The normally asymptomatic human polyomavirus, JCPyV, is the causative agent of a rare but fatal demyelinating disease known as progressive multifocal leukoencephalopathy (PML). Individuals at risk for developing PML include those with AIDS, with other underlying immunosuppressive diseases, and in patients treated with immunomodulatory regimens. Drugs to prevent viral reactivation in the setting of immunosuppression or immunomodulation could be used to sustain lives. Development of such drugs has been impeded by the difficulty of growing and studying the virus. We sought to develop a more efficient method for screening drugs that inhibit viral infection. Pseudovirus models have been developed which may be of use in pharmaceutical research. The use of pseudoviruses as models for viral infection is dependent on them using similar pathways for infection as virus. We screened known inhibitors of viral entry for their ability to block pseudovirus infection. Here we show that the pseudovirus based on the human polyomavirus JCPyV recapitulates virus binding, entry and trafficking. This system can be used for high-throughput screening of antiviral drugs.

1. Introduction

Among the first human polyomaviruses discovered, JCPyV was isolated from the brain of a Hodgkin's lymphoma patient in the early 1970s (Gardner et al., 1971). Although the virus is common, it is asymptomatic in healthy individuals and virus with archetype regulatory arrangement persists in the kidneys. JCPyV causes the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). Disease is associated with the prototypical or PML-type virus that is characterized by rearrangements in the regulatory region (Daniel et al., 1996). This rare brain disorder is due to lytic infection of oligodendrocytes, the cells that provide myelin sheathing for neurons. PML is thought to be due to reactivation of latent virus and occurs only in the setting of immunosuppression. Although PML is still considered a defining disease of AIDS, the incidence has decreased along with advances in highly active antiretroviral therapies (HAART) (Engsig et al., 2009). In the absence of HIV, PML is a rare side effect in patients with other autoimmune diseases such as multiple sclerosis (MS), and hematological disorders, as well as in bone marrow and other transplant recipients (Ferenczy et al., 2012). This risk is increased if the patient's blood tests positive for anti-JCPyV antibodies (FDA, 2012). Recent evidence suggests that PML is becoming more clinically relevant as a widening variety of immunosuppressive treatments are associated with the disease (Schmedt et al., 2012).

Common immunosuppressants such as rituximab, cyclosporine, methotrexate and natalizumab have all been reported as the primary suspected drug in cases of PML. The risk of disease increases over long-term drug treatment. Recent evidence has shown that PML will develop in approximately 1:100 MS patients taking natalizumab, who have been on prior immunosuppressive therapy (Baldwin and Hogg, 2013). Due to lack of alternative MS treatments, when presented with the risks, a small number of patients voluntarily discontinue therapy (van Rossum et al., 2013). Pharmaceutical companies have been forced to stop marketing or selling certain beneficial drugs due to reactivated JCPyV induced PML (FDA, 2006, 2010, 2012; Schmedt et al., 2012). The safety of these drugs could potentially be restored if viral reactivation and spread could be inhibited.

Development of drugs to block polyomaviruses has been hampered by the lack of animal models, and the difficulty of polyomavirus propagation (Mahon et al., 2009; Simon et al., 1999; Steiner and Berger, 2012). In addition, the relative inefficiency of these viruses makes drug screening an arduous task. The efficiency is increased in T antigen (TAg) positive cell lines but the presence of this early viral protein requires infection be scored by measuring late viral protein expression. Restrictions on viral DNA replication and transcription due to its weak promoter further inhibit

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the screening process (Jensen and Major, 2001; Liu and Atwood, 2001). A viral capsid-based model of JCPyV has been developed to study the viral lifecycle, but there is no convenient method of detecting viral infection (Nelson et al., 2012). Pseudovirus models of other polyomaviruses have been developed which have an easier readout and would facilitate the drug screening process (Buck et al., 2005). We have adapted this method to develop a JCPyV pseudovirus model (JCPsV), and have determined that it uses the same pathway as virus to deliver its genome to the nucleus. The JCPsV is easy to grow and provides an efficient model for testing drugs which block binding, entry or viral trafficking.

2. Materials and methods

2.1. Plasmids

Codon optimization of the JCPyV VP1, VP2 and VP3 genes were performed according to the National Cancer Institute Center for Cancer Research Lab of Cellular Oncology Technical Files (http://home.ccr.cancer.gov/LCO/production.asp) in order for optimal expression in the human derived cell line 293FT (Life Technologies Cat # R700-07). The genes were synthesized by Blue Heron Biotech, LLC, Bothell, WA, USA. Sequences were based on the *Polyomaviridae Orthopolyomavirus* strain Mad-1 (NC_001699.1). The VP1 gene was subcloned into the pwP vector in place of the MPyV VP1 gene. The JC VP2 and VP3 genes were subcloned into the ph2p vectors in place of the MPyV genes. (Tolstov et al., 2009) The luciferase reporter vector phGluc (Pastrana et al., 2009) expresses a secreted form of Gaussia luciferase under control of EF1alpha promoter and EGFP under the control of the SV40 promoter. All plasmids were obtained from AddGene (Cambridge, MA, USA).

2.2. Pseudovirus production

Pseudoviruses were produced by transfection of the VP1, VP2, VP3 and phGluc plasmids into 293FT cells using the FuGENE® 6 Transfection Reagent (Promega Madison, WI, USA cat # E2692) in a 5:1:1:1 ratio. As the viral structural proteins are expressed, they assemble into pseudovirus particles and package available plasmid DNA. JCPsV is tittered by calculating the number of viral particles that have packaged the phGluc reporter plasmid. A mock virus preparation prepared using the phGluc vector exactly as pseudovirus but substituting a pUC19 empty vector for the viral protein expressing plasmids. This mock virus was used to control for the presence of phGluc or luciferase co-purifying with pseudovirus.

2.3. Pseudovirus purification

Cells were harvested at 48 h post-transfection by scraping, pelleted and resuspended in Buffer A with protease inhibitors (complete, EDTA-free protease inhibitor cocktail tablets cat # 04693132001, Roche Applied Science Indianapolis, IN, USA). Cells were lysed by three rounds of freezing and thawing, sonicated and treated with .25% deoxycholic acid at 37 °C for 30 min. The pH was lowered to 6.0 and the lysates were treated with type V neuraminidase (Sigma Cat# N2876) at 37 °C for 1 h. The pH was then raised to 7.5, calcium chloride was added and the lysate was treated with DNAse I (New England BioLabs Ipswich, MA, USA cat# M0303S). Pseudoviruses were then purified over an iodixanol gradient, and spun for 3.5 h at 16 °C at 50,000 rpm (234,000 × *g*) in an SW55ti rotor. The band containing pseudovirus was extracted by syringe. For the mock virus, the fraction corresponding to the position where pseudovirus was identified was extracted. Although plasmids up to 9.4 kbp have been shown to be packaged into the larger VP1-only virus like particles (VLPs), viral vectors usually package DNA close to the size of the genome, and it was assumed that one copy of the phGluc reporter plasmid (4463 bp) would be encapsidated into each infectious JCPsV particle (Fang et al., 2012).

2.4. Pseudovirus titration

To titer the pseudovirus with properly encapsidated phGluc plasmid, the purified pseudovirus was again treated with DNAse I to remove any unpackaged plasmid. DNAse I was then inactivated by heating to 75 °C for 10 min. The phGluc reporter plasmid that was protected by the viral capsid from enzymatic degradation was then extracted using the DNeasy Blood & Tissue Kit (Qiagen Valencia, CA USA cat # 69504). The copies of pseudovirus which packaged phGluc were measured using absolute quantification and TaqMan qPCR (Applied Biosystems) to create a standard curve using serial dilutions of phGluc ranging from 15 ng (3 e9 copies) to 1.5 ag (.3 copies) of input DNA. The Ct value for the known amounts of DNA added to the qPCR reaction was plotted against the number of copies of DNA in a scatter plot. The best fit trendline equation from regression analysis was used to calculate the relationship between the Ct value and copies of packaged plasmid. Using this method we could quantify the number of pseudoviruses containing the phGluc reporter plasmid, or genome equivalents (GE) added during each infection.

2.5. Cell lines

293FT cells are derived from human embryonic kidney cells transformed with the SV40 large TAg (Invitrogen Life Technologies), SVGA and SVGR2 cells are subclones of the SV40 origin-defective transformed human glial cell line SVG (Major et al., 1985), SVGR2 cells are resistant to JCPyV infection (Gee et al., 2003), Ntera-2 cells (Pleasure and Lee, 1993) are a pluripotent embryonal carcinoma line, POJ19II (Mandl et al., 1987) are human fetal glial cells transformed with a replication-defective JCPyV. HeLa cells are a human epithelial cell line derived from cervical carcinoma, CV1 and Vero are monkey kidney epithelial cell lines, C8D1A and C8D30 are mouse astrocytic cell lines, NIH3T3 is a mouse embryonic fibroblast cell line, C6 is a rat glial cell line NRK is a rat kidney epithelial cell line and CHO cells are an epithelial cell line derived from hamster ovaries (ATCC, Manassas, VA).

293FT were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented to contain 10% fetal bovine serum (FBS) (Mediatech, Inc.), 0.1 mM non-essential amino acids (NEAA), 6 mM L-glutamine, 1 mM sodium pyruvate, and 500 mg/ml geneticin. Vero cells are grown in MEM with 5%FBS. All other cells were grown in DMEM or MEM supplemented to contain 10% FBS in a humidified incubator at 37 °C and 5% CO_2.

2.6. Infections

Cells were seeded 24 h prior to infection in 96 well dishes at 5000–7000 cells per well. Infections were performed by diluting 10^8 GE/mL in a total volume 35 μL in serum free media and infecting approximately 10,000 cells for 2 h at 37 °C. Cells were washed and media was replaced with phenol red free media and neutralizing JCPyV antibody diluted 1:1000. Viral infection was measured at 72 h post-infection by detection of secreted luciferase in the supernatant using the BioLux® *Gaussia* Luciferase Assay Kit (NEB cat # E3300L). Cell toxicity was measured from the same samples using the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega cat # G5421). Metabolically active cells release a product into the supernatant which is measured by absorbance at 490 nm and is directly proportional to the number of cells. Numbers of infected cells were also be measured by counting GFP positive