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Titration of cell-associated varicella-zoster virus with the MV9G reporter cell line for antiviral studies

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ABSTRACT

Titration of the cell-associated virus (CAV) of varicella-zoster virus (VZV) is essential for antiviral studies. A VZV reporter cell line, MV9G, generated in our previous study expresses firefly luciferase upon CAV infection in a dose-dependent manner, suggesting that use of the cell line for titration is feasible. In this study, MeWo cells infected with VZV vaccine Oka (vOka) strain or with clinical isolates obtained from patients with varicella or zoster were used as CAV. A co-culture of MV9G cells with the virus-infected MeWo cells were set up and optimized for titration of CAV. Luciferase activities of MV9G cells measured as relative light units (RLUs) of chemiluminescence correlated well ($r > 0.9$, $p < 0.05$) both with quantities of viral DNAs measured by TaqMan PCR and with numbers of viral foci detected by immunostaining with a monoclonal antibody against VZV IE62. In addition, the usefulness of MV9G for antiviral studies was exemplified by treatment of the VZV-infected cells with various concentrations of acyclovir. Thus, the reporter cell-based titration of CAV by measuring the induced RLUs may be a reliable way to estimate viral foci and viral DNAs.

1. Introduction

Varicella-zoster virus (VZV) causes varicella (chickenpox) upon primary infection and zoster (shingles) upon reactivation of the latent virus. Nucleoside analogs, including acyclovir, valacyclovir, and famciclovir are effective antiviral drugs for control of VZV infections in current clinical use, which inhibit virus replication in a thymidine kinase-dependent manner (De Clercq, 2004; Arvin and Gilden, 2013). However, resistance of VZV strains to acyclovir has been increasingly reported, especially in transplant recipients (van der Beek et al., 2013; Zuckerman and Limaye, 2013) or HIV-infected individuals (Saint-Léger et al., 2001; Bleymehl et al., 2011; Piret and Boivin, 2014). Although infection with an acyclovir-resistant VZV strain can be treated with foscarnet or vidarabine, which directly target the viral DNA polymerase, these drugs require intravenous administration and have severe side effects, such as renal toxicity or gastrointestinal adverse reactions, which hamper its clinical use (De Clercq, 2004, 2011). Moreover, resistance of VZV strains to foscarnet or to both acyclovir and foscarnet have also been reported (Visse et al., 1998; Bleymehl et al., 2011). These necessitated the screening for novel antiviral agents.

Plaque reduction assay (PRA) and yield reduction assay (YRA) have been widely used as conventional methods in antiviral studies. These assays require enumeration of virus plaques by visual inspection of the infected cell monolayer. Since VZV is a labile and highly cell-associated virus that grows slowly in cell cultures, these methods are not only labor-intensive and time-consuming but also have a limited throughput with high associated variability (Gates et al., 2009). For example, possible selection bias in counting virus plaques and the heterogeneous viral growth speeds in cell cultures can potentially limit the usefulness of PRA or YRA (Piret and Boivin, 2014). To reduce the inconvenience of PRA or YRA, several modified methods have been reported, including immunostaining of the virus-infected cells with monoclonal antibodies (Schmidtmayerová et al., 1986), which is also known as focus reduction assay (FRA) or focus luminescence assay (FLA) (Inoue et al., 2012;

Abbreviations: VZV, varicella-zoster virus; CFV, cell-free virus; CAV, cell-associated virus; PFU, plaque forming unit; RLU, relative light unit; PRA, plaque reduction assay; YRA, yield reduction assay; FRA, focus reduction assay

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Heider and Schroeder, 1997; Borisevich et al., 2008), fluorescence-based antiviral assay (Dal Pozzo et al., 2008), semi-automated flow cytometric assay (Borisevich et al., 2008; Gates et al., 2009), DNA probe assay (Standring-Cox et al., 1996), and TaqMan PCR (polymerase chain reaction, Hawrami and Brueuer, 1999; Furuta et al., 2001). Combined use of PCR with microplate hybridization has been reported to enhance the sensitivity of the quantitative assay of VZV DNA with a minimum detection limit of ten copies (Hondo et al., 1995). Recently, an envelope glycoprotein K (gK)-based neutralization enzyme-linked immunosorbent spot assay (ELISA) and a glycoprotein E (gE) monoclonal antibody-based ELISA quantitative assay have been developed as substitutive methods for PRA (Chen et al., 2014; Liu et al., 2015). However, the lack of a simple and rapid method for titration of VZV has hampered the efforts to screen for new antiviral agents.

To simplify the screening procedures for new antiviral candidates against VZV, a reporter cell line, MV9G, for VZV was generated in our previous study by stable co-transfection of pGL-T9G plasmids along with pCMV-script plasmids into a human melanoma MeWo cell line. The pGL-T9G is a promoter-reporter plasmid encoding a firefly luciferase reporter gene driven by a duplicated tandem repeat of the minimum promoter for VZV ORF9, and the pCMV-script plasmid encodes a G418-resistant gene (Wang et al., 2006). The cells were promoted to express firefly luciferase upon infection of cell-free virus (CFV) by inoculation into culture medium of the MV9G cell monolayer (CFV infection), or upon infection of cell-associated virus (CAV) by co-culture of MV9G cells with virus-infected cells (CAV co-culture). Luciferase activities of MV9G cells shown as relative light units (RLUs) were expressed in a manner dependent on plaque forming units (*pfu*) of CFV infection, and the minimum detection limit of the assay was 50 *pfu* of CFV. CAV infections also promoted MV9G cells to express luciferase in a similar manner with a minimum detection limit of ten virus-infected cells, indicating that the induced RLUs of MV9G cells reflected both CFV titer (*pfu*) and virus-infected cell numbers (Wang et al., 2006). With this reporter cell-based antiviral assay, a novel anti-VZV compound, 35B2, has been identified from a library of 9600 random compounds. The compound showed an antiviral mechanism targeting the assembly of the major capsid protein of VZV (Inoue et al., 2012), which is different from those of the antiviral drugs in current clinical use (De Clercq, 2004). A similar reporter cell line for human cytomegalovirus has also been successfully generated and applied to the screening for antiviral compounds (Fukui et al., 2008). However, it is uncertain yet whether the induced RLUs of MV9G cells in the co-culture assay correlate with numbers of viral foci or with amounts of viral DNA of the co-cultured CAV.

In this study, human melanoma MeWo cell lines infected with VZV clinical isolate or with vaccine strain were used as CAV, co-culture of MV9G cells with CAV was set up and optimized, and usefulness of the reporter cell line MV9G in titration of CAV was validated.

2. Materials and methods

2.1. Cells and viruses

The human melanoma MeWo cell line, granular fibroblasts derived from a human malignant melanoma which support the growth of VZV isolates (Grose and Brunel, 1978), were purchased from the American Type Culture Collection (ATCC HTB-65, VA, U.S.A.). MeWo cells were grown and propagated in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml streptomycin. The reporter cell line MV9G, a MeWo cell line stably co-transfected with plasmids encoding firefly luciferase reporter gene along with plasmids encoding G418-resistant gene, was grown and propagated in the medium supplemented with 0.2 mg/ml of G418. A VZV clinical isolate, Z44, obtained from vesicular lesions of a patient with zoster, another clinical isolate V23 obtained from vesicular lesions of a patient with varicella, and a vOka strain provided by Changchun BCHT Biotechnology, Co. of China were grown on MeWo cells. VZV was identified by immunohistochemical staining with a monoclonal antibody against VZV immediate early 62 (IE62) antigens (MAB8616, 1 : 500, Chemicon, CA, U.S.A.). The vOka strain was genetically verified by the assay described previously (Gomi et al., 2002) (data not shown). MeWo cells infected with the clinical isolates Z44, V23, or vOka strain were used as CAV in this study. Reagents for cell culture and virus propagation were purchased from Gibco-Invitrogen (CA, U.S.A.).

2.2. Antiviral chemicals

Acycloguanosine (acyclovir) was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China) with a purity > 99.0%.

2.3. CAV co-culture

MV9G cells (9×10^4/well) were seeded in triplicate wells of 96-well plates and cultured for approximately 24 h to achieve confluence. Additional triplicate control wells were set up in parallel for cell number counting. When virus-infected MeWo cells in a T-25 flask reached approximately 50% of their cytopathic effects (CPE), the cells were harvested, and the cell pellets were washed twice with phosphate-buffered saline (PBS) to remove cell-free viruses, and then suspended with culture medium. After counting cell density, the virus-infected cells were immunohistochemically stained with an IE62 monoclonal antibody (MAB8616, Chemicon) and visualized by diaminobenzidine (DAB) staining on cell suspension smear. The numbers of IE62-positive and -negative cells were counted under a microscope, and the percentage of IE62-positive cells was calculated. CAV preparations diluted based on the calculated percentage of virus-infected cells were overlaid onto a MV9G cell monolayer for co-culture for an additional 48 h.

2.4. Luciferase activities test

MV9G cells (9×10^4/well) were seeded in 96-well plates and cultured for approximately 24 h to form a monolayer. MeWo cells infected with VZV clinical isolate or vOka strain reaching 50% CPE were plated onto a monolayer of MV9G cells at a multiplicity of infection (MOI) of 0.1 and co-cultured for 48 h in the absence or presence of acyclovir. Before plating, the cell pellets were washed twice with PBS to remove cell-free viruses as described above. After co-culture, the cells were harvested, and the induced RLUs of MV9G cells were measured with a Steady-Glo ® Luciferase Assay kit (Promega, WI, U.S.A.) with a minor modification. Briefly, after removal of culture media, the cells were rinsed with PBS and then frozen at −80 °C for 30 min. After thawing at room temperature, the cells were frozen again at −80 °C for 30 min with the addition of 25 μl/well of PBS. When the frozen cells melted at room temperature, 25 μl/well of Steady-Glo ® reagents were added to the cell lysates and incubated at room temperature for 45 min in a dark place. Thirty microliters of the reaction mixture were then transferred into black 96-well plates (Corning, NY, U.S.A.), RLUs of MV9G cells were measured with GloMax®-Multi Chemiluminescence Detector (Promega).

2.5. Viral foci enumeration

Virus-infected MeWo cells were plated onto a monolayer of MV9G cells at an M.O.I. of 0.1 and co-cultured for 48 h in the absence or presence of acyclovir. Free viruses not in the infected cells were removed as described above. After removal of PBS, cells were fixed with 5% formalin for 5 min and then treated with 0.5% Triton-X100 for 10 min. Thirty microliters per well of monoclonal antibody against VZV IE62 (1:500, Chemicon) were added and incubated at 4 °C overnight in a humid chamber, then 30 μl/well of horseradish peroxidase-labeled sheep-anti-mouse immunoglobulin G (IgG) antibodies (1:2, 000, ZSGB Bio., Beijing, China) were added and incubated at 37 °C for 60 min in a