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Infected T98G glioblastoma cells support human cytomegalovirus reactivation from latency

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ABSTRACT

T98G cells have been shown to support long-term human cytomegalovirus (HCMV) genome maintenance without infectious virus release. However, it remains unclear whether these viral genomes could be reactivated. To address this question, a recombinant HCMV (rHCMV) containing a GFP gene was used to infect T98G cells, and the infected cells absent of infectious virus production were designated T98G-LrV. Upon dibutyryl cAMP plus IBMX (cAMP/IBMX) treatment, a serial of phenomena were observed, including GFP signal increase, viral genome replication, lytic genes expression and infectious viruses release, indicating the reactivation of HCMV in T98G-LrV cells from a latent status. Mechanistically, HCMV reactivation in the T98G-LrV cells induced by cAMP/IBMX was associated with the PKA-CREB signaling pathway. These results demonstrate that HCMV was latent in T98G-LrV cells and could be reactivated. The T98G-LrV cells represent an effective model for investigating the mechanisms of HCMV reactivation from latency in the context of neural cells.

1. Introduction

Human cytomegalovirus (HCMV) is a member of the beta-herpesvirinae. It is a ubiquitous pathogen with the serum positive rate as high as 95% in China. In immunocompetent individuals, primary HCMV infection is typically asymptomatic, but establishes a lifelong persistent/latent infection in its host. However, in immunocompromised individuals, such as AIDS patients and transplant recipients, primary infection or reactivation/recurrent infection of HCMV results in serious diseases, including pneumonia, gastrointestinal disease, retinitis and nephritis (Ljungman et al., 2002). In addition, congenital HCMV infection caused by a maternal primary-infection or reactivation causes 5–10% of infected neonates to suffer microcephaly or periventricular calcification at birth. 10–15% of the subclinically infected infants subsequently develop late-onset sequelae including sensorineural hearing loss, mental retardation and learning disabilities within 3 years (Leung et al., 2003). HCMV reactivation has largely focused on hematopoietic stem cells (Goodrum et al., 2002; Hahn et al., 1998a; Khaiboullina et al., 2004; Kondo et al., 1994; Mendelson et al., 1996; Soderberg-Naucler et al., 2001). Certainly late-onset neurodevelopmental disorders could be caused by virus reactivation from the myeloid reservoirs, persistent infection, or a new lytic infection. However, were they to exist in vivo, reactivation from latently infected neural cells could also contribute. In order to study the potential mechanism(s) of late-onset neurodevelopmental disorders caused by congenital HCMV infection, an effective latent-reactivation HCMV infection model in the context of neural cell type is crucial.

HCMV infection is characterized as lytic or persistent/latent infection. During lytic infection in permissive cells (such as fibroblasts, endothelial cells, epithelial cells and macrophages), viral genes are expressed in an temporal cascade (Wathen et al., 1981; Wathen and Stinski, 1982). The major immediate early (IE) genes are the first viral genes to be transcribed, resulting in abundant proteins (such as IE1 and IE2). These IE proteins activate the expression of early genes (such

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as UL44 and UL54), which are required for viral DNA replication and eventually lead to the expression of late genes (such as UL94 and UL99). Finally, the progeny viruses are assembled and released.

After primary infection, HCMV establishes a latent infection in specific sites within the hosts. Latent infection is defined as a type of persistent infection and characterized as maintenance of the viral genome without shedding infectious virus except for intermittent episodes of reactivation (Knipe et al., 2013). At present, latent HCMV is commonly accepted to reside within hematopoietic stem cells in vivo, particularly in undifferentiated myeloid lineage and monocytes, such as $CD34^+$ progenitor cells (Hahn et al., 1998b), granulocyte-macrophage progenitors (GM-Ps) (Kondo et al., 1994), and $CD14^+$ monocytes (Bolovan-Fritts et al., 1999; Taylor-Wiedeman et al., 1991). *In vitro* HCMV latent cell models, including embryonic stem cell lines (Penkert and Kalejta, 2013), myeloid progenitor cell line Kasumi-3 (O'Connor and Murphy, 2012), monocytic THP-1 cells (Bego et al., 2005; Weinshenker et al., 1988), and human teratocarcinoma Nera-2 (NT2) cells (Gonczol et al., 1984), have been well established. These models are useful for exploring HCMV pathogenesis in immunocompromised individuals, but not suitable for investigating the mechanism of HCMV reactivation in the context of neural cells.

Our previous studies have demonstrated that T98G glioblastoma cells are semi-permissive for HCMV infection as viral protein expression is delayed. Moreover, HCMV-infected T98G cells harbor viral genomes but without detectable infectious virus following passaging (Duan et al., 2014; Luo and Fortunato, 2007). This suggested the T98G cells might serve as an HCMV latent-infection cell model. However, the latency status of HCMV in T98G cells can only be confirmed upon successful reactivation, evidenced by viral replication and release of infectious viruses, which remains unclear so far.

The stimuli and the corresponding mechanisms involved in HCMV reactivation are not fully understood. Previous reports demonstrate that HCMV lytic infection is dependent on the status of cellular differentiation. Treatments with cellular differentiation associated agents, such as phorbol ester (TPA), retinoic acid, cyclic AMP (cAMP) or cAMP plus 3-isobutyl-1-methylxanthine (IBMX), resulted in differentiating cells into HCMV-permissive cells, inducing the viral lytic genes transcription and/or promoting infectious virions release (Matsukage et al., 2006; Meier, 2001; Poland et al., 1994; Stamminger et al., 1990; Weinshenker et al., 1988). Moreover, it is becoming increasingly evident that chromatin remodeling (as histone modifications present on the MIEP, resulting in chromatin structure loosening) affects the transcriptional activity in HCMV reactivation (Ioudinkova et al., 2006; Reeves, 2011; Sinclair, 2010). A series of inhibitors specific for epigenetic regulation, including histone deacetylase (HDACs) inhibitors trichostatin A and sodium butyrate, and DNA methylation inhibitor 5′-aza-2′- deoxycytidine, have been proved to be capable of activating the CMV promoter (Choi et al., 2005; Grassi et al., 2003; Murphy et al., 2002).

Here we used a Towne strain derived recombinant HCMV (rHCMV) containing an SV40 promoter driven GFP gene in the viral genome. rHCMV can be visualized by fluorescence microscopy and analyzed by flow cytometry, allowing determination of rHCMV infection status in the T98G cells. The infected cells absent of infectious virus production were designated as T98G-LrV. We further observed that dibutyryl cAMP plus IBMX (cAMP/IBMX) treatment effectively reactivated the latent rHCMV in T98G-LrV potentially via the PKA-CERB signaling pathway. This cell model of brain origin should offer a significant improvement to understand the pathogenesis of HCMV-caused late-onset neurodevelopmental disorders.

2. Materials and methods

2.1. Cells culture and virus infection

Human T98G glioblastoma cell line (ATCC CRL-1690) and human foreskin fibroblasts (HFFs) were cultured in growth medium of minimal essential medium (MEM) (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL).

Recombinant HCMV virus (rHCMV) was derived from Towne strain by inserting a GFP gene driven by an SV40 promoter into the viral genome (Marchini et al., 2001). Virus was propagated in HFFs and virus titers were determined by plaque forming assay as described previously (Compton et al., 1992).

T98G cells were infected with rHCMV as previously reported (Duan et al., 2014; Luo and Fortunato, 2007). Briefly, serum starved T98G cells were plated in 100 mm cell culture dishes (5×10^5 cells/dish) and infected with rHCMV at an multiplicity of infection (MOI) of 10. At 16 h post infection, the inoculum was removed, and the cells were refed with growth medium after washing with phosphate-buffered saline (PBS, pH 7.4). Then the infected T98G cells were reseeded to new 100 mm dishes at a density of 5×10^5 cells/dish, cultured for 7 days and passaged. Notably, the interval of passaging was modified to 7 days instead of 3 days as previously reported, which is to ensure the virus completing a full replication cycle. The original rHCMV-infected T98G cells were designated as passage 0 (P0), the following subculture cells were designated as P1, P2,..... Cells at P5 which maintained viral genome with undetectable infectious virus were designated as T98G-LrV cells.

2.2. Treatment with chemical reagents

T98G-LrV cells were plated in T25 flasks (5×10^5 cells/flask) or 6-well plates (1.5×10^6 cells/plate). To reactivate the latent virus in T98G-LrV cells, the growth medium was replaced with maintenance medium of MEM supplemented with 2% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), 100 μM dibutyryl cAMP, and 100 μM IBMX. The cells were cultured in the presence of the treatment reagents with refreshing the medium every 4 days, and harvested until the indicated times. Where indicated, T98G-LrV cells were pretreated with phosphonoacetic acid (PAA, 50 μg/ml) and H89 (5 μM or 10 μM). All chemical reagents used in the study were purchased from Sigma (Sigma-Aldrich, USA).

2.3. Determination of infectious virus

Supernatant samples or cells were harvested. The cell pellets were resuspended in the growth medium. Aliquots of the cell suspension were repeatedly freeze-thawed to prepare cell lysate samples. The supernatants and cell lysates were inoculated on monolayers of HFFs to evaluate the infectious virus which was judged by GFP signals. At 14 days post incubation, the cells were observed with a Nikon Eclipse Ti-S fluorescence microscope (Nikon, Tokyo, Japan).

After reactivation treatment, infectious cell-associated viruses were determined by coculture HFFs with treated cells directly or after sonication. DMSO or cAMP/IBMX treated T98G-LrV cells were harvested, and resuspended to 1×10^5 cells/ml. A portion of the cell suspension was sonicated to prepare the sonicated cell samples. Treated T98G-LrV cells (1×10^5) or the sonicated cells (1×10^5) were inoculated onto monolayers of HFFs (5×10^5). Three hours later, soft agar (0.4% W/V) was added to characterize the ability of cells to form GFP plaques. After 10 days post coculture, GFP plaques on HFFs were observed with a Nikon Eclipse Ti-S fluorescence microscope (Nikon, Tokyo, Japan).

2.4. Flow cytometry (FACS)

Aliquots of cells were harvested after trypsinization and resuspended in PBS. GFP-positive cells were determined on a FACScan flow cytometer using the CellQuest software package (Becton Dickinson, Franklin Lakes, N.J.). Twenty thousand live cell events