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Relevance of non-synonymous thymidine kinase mutations for antiviral resistance of recombinant herpes simplex virus type 2 strains

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ABSTRACT

Therapy or prophylaxis of herpes simplex virus type 2 (HSV-2) infections with the nucleoside analog aciclovir (ACV) can lead to the emergence of drug-resistant HSV-2 strains, particularly in immunocompromised patients. In this context, multiple amino acid (aa) changes can accumulate in the ACV-converting viral thymidine kinase (TK) which hampers sequence-based diagnostics significantly. In this study, the so far unknown or still doubted relevance of several individual aa changes for drug resistance in HSV-2 was clarified. For this purpose, ten recombinant fluorescent HSV-2 strains differing in the respective aa within their TK were constructed using the bacterial artificial chromosome (BAC) pHSV2(MS)Lox. Similar TK expression levels and similar replication behavior patterns were demonstrated for the mutants as compared to the unmodified BAC-derived HSV-2 strain. Subsequently, the resulting strains were tested for their susceptibility to ACV as well as penciclovir (PCV) in parallel to a modified cytopathic effect (CPE) inhibition assay and by determining the relative fluorescence intensity (quantified using units, RFU) as a measure for the viral replication capacity. While aa changes Y53N and R221H conferred ACV resistance with cross-resistance to PCV, the aa changes G25A, G39E, T131M, Y133F, G150D, A157T, R248W, and L342W maintained a susceptible phenotype against both antivirals. The CPE inhibition assay and the measurement of relative fluorescence intensity yielded comparable results for the phenotypic testing of recombinant viruses. The latter test showed some technical advantages. In conclusion, the significance of single aa changes in HSV-2 TK on ACV/PCV resistance was clarified by the construction and phenotypic testing of recombinant viral strains. This was facilitated by the fluorescence based method.

1. Introduction

Herpes simplex virus type 2 (HSV-2) is a species of the genus *Simplexvirus* of the subfamily *Alphaherpesvirinae* within the *Herpesviridae* family (Davison, 2010). This DNA virus is predominantly transmitted by sexual intercourse and is considered the major cause of genital herpes (Smith and Robinson, 2002). Following primary infection, latency is established in sensory ganglia. Viral reactivation occurs frequently causing recurrent episodes of symptomatic genital herpes or asymptomatic virus shedding (Hofstetter et al., 2014). Both can lead to HSV-2 transmission to sexual partners but also to neonates (Johnston and Corey, 2016; Sauerbrei, 2016). Global HSV-2 seroprevalence is higher in women (14.8%) than in men (8%), and 19.2 million incident infections occur each year (Johnston and Corey, 2016). HSV-2 infection represents a marked risk for acquisition or transmission of human immunodeficiency virus (HIV) infection (Freeman et al., 2006).

So far, prophylactic or therapeutic HSV-2 vaccines are not available (Sauerbrei, 2016). Nucleoside analogs such as aciclovir (ACV) or its oral prodrug valaciclovir (VACV) are currently the mainstay of therapy. These acyclic guanosine analogs act highly specifically since monophosphorylation is selectively mediated by the viral thymidine kinase (TK, *UL23*). Subsequently, cellular kinases lead to the active triphosphorylated compound which inhibits viral DNA polymerase resulting in DNA chain termination (Gilbert et al., 2002). Structurally related compounds such as penciclovir (PCV) do not lack the second hydroxy group and hamper DNA replication by retarding further nucleotide

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incorporation (De Clercq, 2013). Viral strains with resistance to ACV/VACV are normally cross-resistant to PCV and its oral prodrug famciclovir (FCV) (Sauerbrei et al., 2016). Under these conditions, foscarnet (FOS) represents the first-choice alternative drug for antiviral treatment (Khurana et al., 2005; Sauerbrei et al., 2011). This pyrophosphate analog does not need to be activated through phosphorylation, and, therefore, affects viral DNA polymerase directly (Crumpacker, 1992). In addition, cidofovir (CDV), an acyclic nucleoside phosphonate analog, is also able to directly inhibit DNA polymerase activity (Naesens et al., 1997).

Genital herpes can be associated with resistance to ACV, VACV, PCV, and FCV, especially in immunocompromised patients as result of extended viral replication and the reduced elimination of resistant mutants (Piret and Boivin, 2011). Thus, ACV-resistant HSV-2 strains have been observed in up to 5% of HIV-positive patients (Danve-Szatanek et al., 2014; Reyes et al., 2003). The vast majority of resistance-related amino acid (aa) changes are located in the TK as a result of non-synonymous or frameshift mutations as well as extra stop codons which occur mostly in conserved gene regions (Bohn-Wippert et al., 2015; Sauerbrei et al., 2016). However, the polymorphic nature of the TK gene (Bohn et al., 2011) requires the examination of each aa change for its relation to resistance (Sauerbrei et al., 2016). For HSV-2 polymerase, at least 17 resistance related aa changes and one premature stop of translation have been published to 2016 (Sauerbrei et al., 2016).

Previously, we studied the impact of HSV-1 and varicella-zoster virus (VZV) TK mutations on antiviral resistance by applying *en passant* mutagenesis (Brunnemann et al., 2015, 2016). In the present study, this technique was used to examine ten TK aa changes (G25A, G39E, Y53N, T131M, Y133F, G150D, A157T, R221H, R248W, and L342W) which all have been recently detected in clinical HSV-2 strains (Bohn-Wippert et al., 2015; Burrel et al., 2010; Mitterreiter et al., 2016; Muller et al., 2013; Sauerbrei et al., 2010, 2011).

2. Material and methods

2.1. Amino acid changes of HSV-2 thymidine kinase

The aa changes G25A, Y53N, Y133F, and R221H were observed in the TK of ACV-resistant clinical HSV-2 isolates (Sauerbrei et al., 2010; Burrel et al., 2010) and were, therefore, potentially associated with ACV resistance. The four additional aa changes G150D, A157T, R248W, and L342W have been described as novel natural polymorphisms in clinical HSV-2 strains by Bohn-Wippert et al. (2015). The aa change T131M was first detected by Muller et al. (2013). However, its significance remained unclear until Bohn-Wippert et al. (2015) reported this aa change as part of natural gene polymorphisms in an ACV-susceptible clinical HSV-2 isolate. Finally, the aa change G39E is well-known as caused by a natural TK gene polymorphism (Burrel et al., 2010). However, it has also been reported on two ACV-resistant HSV-2 isolates expressing G39E as single aa change within TK in the presence of further natural polymorphisms in the DNA polymerase (Sauerbrei et al., 2011). Furthermore, a case of an ACV-resistant HSV-2 encephalitis probably related to aa change G39E was previously published (Mitterreiter et al., 2016).

2.2. Generation, replication kinetics, and gene expression analysis of recombinant HSV-2 variants

The generation of recombinant HSV-2 variants containing one of ten non-synonymous TK mutations was enabled by the bacterial artificial chromosome (BAC) pHSV2(MS)Lox which originated from the genomic DNA of the HSV-2 strain MS (ATCC® VR-540DQ™) cloned into pBeloBAC11 (Meseda et al., 2004). The final construct harbors loxP sites as well as the Cre recombinase gene driven by an eukaryotic promoter (Nagel et al., 2008; Nygårdas et al., 2013; Smith and Enquist, 2000) and was maintained in the *E. coli* strain GS1783 to allow *en passant* mutagenesis (Tischer et al., 2006, 2010).

This BAC was supplemented with the enhanced green fluorescent protein (EGFP) gene and consequently designated pHSV2(MS)Lox-GFP (for details see Brunnemann et al., 2016). The *UL23* gene was substituted by a modified gene as described previously (Brunnemann et al., 2016). For this purpose, the plasmid pJET1.2 (ThermoFisher Scientific, Waltham, MA, USA) was used for cloning the blunt-ended amplified native *UL23* gene together with a kanamycin resistance cassette. The ten single nucleotide substitutions causing the respective aa changes (G25A, G39E, Y53N, T131M, Y133F, G150D, A157T, R221H, R248W, and L342W) were then introduced by site-directed mutagenesis (GeneArt™ Site-Directed Mutagenesis System, ThermoFisher Scientific). The *UL23*-kanamycin cassette was amplified with oligonucleotides harboring a 5' overhang of 50 nucleotides homologous to the integration site on the HSV-2 *UL23*-deletion BACs. This modified *UL23* gene was inserted by homologous recombination according to the *en passant* mutagenesis (Brunnemann, 2016; Brunnemann et al., 2016; Tischer et al., 2006). All intermediate and final BAC constructs were checked by restriction fragment length polymorphism and sequencing of the transition regions (data not shown).

The BAC DNA was transfected by Lipofectamine 2000 (ThermoFisher Scientific) into permissive Vero cells (ATCC® CCL-81™) as published in detail by Brunnemann et al. (2016). Virus reconstitution occurred within 3 d. The recombinant HSV-2 strains were amplified by 8–11 cell passages and finally checked by sequencing of the *UL23* gene (data not shown). The replication competence of each strain was compared to the unmodified BAC-derived HSV-2 and its fluorescent counterpart in multi-step replication kinetics performed in triplicate wells with a multiplicity of infection (MOI) of 0.001 over a 72 h period. For this, infected cells were scraped at different time points into medium which was then deep frozen. For titration, the suspension was thawed, cleared by centrifugation and used for infection of fresh Vero cells. Resulting plaques of non-fluorescent strains were read out after crystal-violet staining while plaques of fluorescent strains were checked using fluorescence microscopy (IX80 Olympus, Hamburg, Germany). Viral titers were plotted as the mean and standard deviation (SD) from triplicates in plaque-forming units per ml against the time (Brunnemann, 2016; Brunnemann et al., 2016).

Transcription of the modified *UL23* gene was analyzed at 2 d post infection (p.i.) by reverse transcription (RT) polymerase chain reaction (PCR) applying oligonucleotides specific for HSV-2 TK gene (fw 5′-CAG TAA GTC ATC GGC TCG G-3′; rev 5′-TTC GGT CAG GCT GCT CGT G-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) exon according to Brunnemann et al. (2016). Expression of TK protein in infected Vero cells was verified at 2 d p.i. by immunoblotting and immunofluorescence following Brunnemann et al. (2016).

2.3. Susceptibility testing of recombinant viral strains

Susceptibility testing of *UL23*-modified HSV-2 strains to the antivirals ACV (GlaxoSmithKline, Uxbridge, UK) and PCV (GlaxoSmithKline) was performed in comparison to the unmodified BAC-derived HSV-2 strain which is based on the sensitive reference MS. For sake of completeness all strains were also tested against FOS (AstraZeneca, Wilmslow, UK) and cidofovir (CDV, Vistide®, Pharmacia & Upjohn, Luxembourg). The cytopathic effect (CPE) inhibition assay modified by the use of red tetrazolium dye (Schubert et al., 2014) was used as standard test. As all test viruses, including the reference control, contained an EGFP cassette which is expressed in infected cells, the susceptibility was also examined by direct measuring the relative fluorescence intensities (quantified using units, RFU) in infected cells. While human fetal lung fibroblasts (HELF) were used for susceptibility testing of ACV, PCV, and CDV, Vero cells (ATCC® CCL-81™) were applied for FOS. Conditions for cell cultivation and viral growth were described previously (Brunnemann et al., 2016; Sauerbrei et al., 2010). In short, cells were seeded at a density of 1×10^5 cells ml^{-1} in 96-well

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