Analysis of thymidine kinase mutations conferring acyclovir resistance in herpes simplex type 1 recombinant viruses

Running title: Thymidine kinase mutations

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Abstract: 49
Text: 1331

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Abstract

Contribution of thymidine kinase (TK) mutations to acyclovir (ACV) resistance were evaluated in herpes simplex type 1 recombinant viruses generated using a set of overlapping cosmids and plasmids. Alterations in both conserved and non-conserved regions of the TK gene were shown to confer high levels of resistance to ACV.
Herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) infections are associated with serious complications in immunocompromised hosts. Prolonged antiviral treatment is often required for the clinical management of these patients, which could lead to the emergence of drug-resistant viruses (5, 17). Acyclovir (ACV), which has been the standard treatment for HSV infections, must be first phosphorylated by the viral thymidine kinase (TK) and then by cellular kinases before inhibiting the virus-encoded DNA polymerase (DNA pol) (reviewed in (7)). In clinic, HSV resistance to ACV is most commonly due to mutations (substitutions, deletions or additions) in the viral TK gene (6). Single base substitutions in the viral DNA pol gene have been more rarely reported in ACV-resistant clinical HSV isolates, with or without additional TK mutations (14, 17). Because of the presence of multiple mutations within these two genes including several ones associated with viral polymorphism (2, 9-11, 13, 17), it has been difficult so far to analyze the contribution of each specific HSV mutation with regard to the ACV resistance phenotype. We recently reported the generation and characterization of HSV-1 DNA pol mutants (1) using a set of four overlapping cosmids and three plasmids based on the original work of Cunningham et al. (3). Herein, we report a modification of this cosmid-based recombination system to evaluate the impact of HSV-1 TK mutations as well as double TK-DNA pol mutations on the ACV resistance phenotype.

Vero cells were maintained in minimum essential medium supplemented with 10 % fetal calf serum. The HSV-1 recombinant strain 17 and all mutant viruses derived from this strain were propagated in Vero cells. The method used to generate HSV-1 recombinant viruses from overlapping cosmids (gift of Charles Cunningham, MRC Virology Unit, Glasgow, United Kingdom) as well as the plasmid used for site-directed mutagenesis of the viral DNA pol gene have been described elsewhere (1). Additional modifications were made to this system to introduce TK mutations as follows. One of the 5 viral fragments (cosmid 71, corresponding to nucleotides (nt) 40966 to 77049) was first replaced by three overlapping
plasmids (pNEB23, pPol6, and pNEB10) (1) (Fig. 1). Plasmid pYS-1 was subsequently created by inserting the purified pNEB23 BbvI - MfeI digested fragment (nt 43981 to 49891) into plasmid pSP72 (Promega, Madison, WI), in which BbvI - MfeI restriction sites had been added (Fig. 1). Specific mutations in the TK gene (located between nt 186 to nt 1007 of the viral gene) were introduced into plasmid pYS-1 using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutated BbvI – MfeI fragments were then cloned back into pNEB23. The correct nucleotide sequence of each mutated TK gene was verified by sequence analysis and compared to that of strain 17. Mutations were introduced in the viral DNA pol gene by performing site-specific mutagenesis on plasmid pPol6 as reported (1).

Generation of HSV-1 TK and/or DNA pol mutants, antiviral susceptibility assays and in vitro replication kinetic experiments were done as previously described (1). Briefly, digested cosmids (n = 4) and plasmids (n = 3) were transfected using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) into Vero cells seeded in 12-well plates. Viral cytopathic effects were observed approximately 3-4 days after transfection. The TK and DNA pol sequences of each recombinant virus were again verified by DNA sequencing. Acyclovir susceptibility of each recombinant virus was determined by the use of a plaque reduction assay (PRA) (15). A two-fold difference in IC50 values between the recombinant wild-type (WT) and mutant viruses was considered significant (1). All experiments were done in duplicate using two independently-generated viruses for each mutation of interest. Replication kinetics of each recombinant mutant virus (tested in duplicate at a multiplicity of infection (MOI) of 2) were compared to that of the recombinant WT virus by titrating extracellular viruses collected at specific times post-infection (up to 120 h) onto Vero cells (1).
Eight recombinant HSV-1 viruses (WT, TK-deleted, 4 TK mutants, 1 DNA pol mutant and 1 dual TK-DNA pol mutant), selected on the basis of their report in previous clinical or laboratory studies, were successfully generated in this study. Acyclovir IC$_{50}$ values are reported in Table 1. All recombinant mutants were resistant to ACV with the single DNA pol mutant D907V being the less resistant (4-fold change in IC$_{50}$ compared to WT) and the double TK (deleted C467 with truncated protein)-DNA pol (D907V) mutant being the most resistant (62-fold change in IC$_{50}$). As shown in Fig. 2, the replication kinetics of the mutants were relatively similar to that of the WT virus up to 120 h post-infection.

Although the definition of ACV resistance used in this study was somewhat arbitrary ($\geq$ two-fold change in IC$_{50}$ compared to WT), our results are in agreement with the ACV resistance phenotype previously reported in other studies that most commonly used a resistance cutoff of 2 $\mu$g/ml for clinical isolates (6, 17). Our results obtained with recombinant viruses, which allows mutational studies in a homogeneous background, revealed that several mutations in conserved but also in non-conserved regions of the TK gene are associated with high levels of ACV resistance. The mutation K62N, located in the ATP-binding site of the TK, conferred the highest level of resistance to ACV with the exception of the TK-deleted virus and the dual TK-DNA pol mutant. The importance of the lysine at position 62 has been previously demonstrated in a site-directed mutagenesis study (12). This mutation, also described in an ACV-resistant varicella-zoster virus isolate (19), has been associated with a TK-low producer phenotype. The mutation C336Y, implicated in the conformation of the TK protein, also resulted in a phenotype highly resistant to ACV. The latter mutation was first described in laboratory-derived ACV-resistant mutants (4, 8) but also in a TK-altered clinical HSV-2 isolate (16). Of interest, the mutation P131S, located in a non-conserved region of the TK gene, also conferred an ACV-resistant phenotype. This mutation has been previously described in a laboratory-derived mutant passed in the presence of ACV (18). Since this
ACV-resistant mutant contained both the P131S TK mutation as well as a DNA pol mutation (A719V), our study was able to confirm the role of the former mutation in conferring resistance to ACV. A single TK mutation (deletion of a C at position 467 leading to a truncated protein) induced high level of ACV resistance whereas the single DNA pol mutation D907V, within a non-conserved gene region, induced low level of resistance to ACV. The recombinant virus containing the two previous mutations had the highest ACV IC$_{50}$ value among all recombinant viruses confirming the synergistic effect of dual TK-DNA pol mutations. The simultaneous presence of these two mutations was reported in a HSV-2 isolate from a patient with AIDS in whom ACV and foscarnet therapy sequentially failed (17). Our study unequivocally shows that the deletion of a C at position 467 in a homopolymer run, which introduces a stop codon 25 amino acids downstream, was mainly responsible for the ACV resistance phenotype of this isolate. Finally, as expected, the TK-deleted recombinant virus was highly resistant to ACV although not much more than single TK substitutions such as K62N and C336Y.

Alterations in the TK are most frequently seen in clinic, probably because this protein is not essential for viral replication in most tissues and cultured cells (6, 13). Thus, it is not expected that mutations in this gene would affect viral growth kinetics. Indeed, the replication kinetics of all tested recombinant viruses including the TK-deleted virus were similar to that of the WT virus. In addition, mutation D907V, located in a non-conserved region of the DNA pol gene, did not affect viral replication in contrast to other previously-reported DNA pol mutations located within conserved gene regions (1).

In conclusion, we have developed a system that now allows rapid assessment of any mutations found in both viral genes associated with ACV resistance. Such methodology could
serve to develop virtual drug phenotypes for HSV with systematic evaluation of viral mutations found in clinical specimens.
References:


**Table 1.** Phenotypic and genotypic analyses of thymidine kinase and/or DNA polymerase HSV-1 recombinant mutants.

<table>
<thead>
<tr>
<th>TK mutation</th>
<th>DNA pol mutation</th>
<th>Gene locations</th>
<th>ACV IC&lt;sub&gt;50&lt;/sub&gt; values in µg/ml* (fold-change vs WT)</th>
<th>References</th>
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<tr>
<td>---</td>
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<td>nt</td>
<td>Aa</td>
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<td>Deleted C 467</td>
<td>Stop 25 aa ds</td>
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<td>A3735T</td>
<td>D907V</td>
<td>Between region I and VII of DNA pol</td>
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*Acyclovir IC<sub>50</sub> values were obtained from two independently-generated recombinant viruses, each tested in duplicate.

Note. TK, thymidine kinase; pol, polymerase; ds, downstream; nt, nucleotide; aa, amino acid; ACV, acyclovir; IC<sub>50</sub>, 50% inhibitory concentration; WT, wild-type.
Figure legends:

**Figure 1:** Schematic representation of viral cosmids and plasmids used to generate HSV-1 recombinants into Vero cells. (A) Digested fragments of five cosmids (24, 32, 48, 51 and 71) covering the complete genome of HSV-1 strain 17 (3) with three plasmids (pNEB23, pPol 6 and pNEB10) designed to replace cos 71. (B) Construction of plasmids pNEB23, pPol 6, pNEB10 and (C) pYS-1.

**Figure 2:** Replication kinetics of several HSV-1 mutants and the WT virus in a single-step growth experiment. On the day of infection, confluent Vero cells in 12-well plates were infected with recombinant viruses at a MOI of 2. Extracellular viruses were collected at different times post-infection (2, 18, 24, 36, 48, 72, 96 and 120 h) and titrated onto Vero cells. Curves represent mean of two independent experiments. A: WT and single TK recombinant mutants, B: WT and dual TK-DNA pol recombinant mutants (alone and in combination).
PCR-amplified and Xba I/Hind III digested fragment (6 Kb) from cos 71

A

Tr
UL
UL 23
UL 30
Ir
Ir
US
Tr

TK
DNA pol

Xba I
Hind III

B

Xba I
Hind III

C

pYs-1 (pSP72)

pNEB23
pNEB10

Cos 71 Pac I / Psi I-digested fragment (23 Kb)
Cos 71 Pac I / Kpn I-digested fragment (10 Kb)

Cos 71 Pac I / Psi I-digested fragment (23 Kb)
Cos 71 Pac I / Kpn I-digested fragment (10 Kb)
A

B

viral titer (mean log pfu/ul)

viral titer (mean log pfu/ul)

hours post-infection

hours post-infection

WT

P131S (TK)

K62N (TK)

C336Y (TK)

TK- (TK)

D907V (DNA pol)

D907V/C467 (DNA pol-TK)

C467 (TK)

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