Latent Acyclovir-Resistant Herpes Simplex Virus Type 1 in Trigeminal Ganglia of Immunocompetent Individuals

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Specific mutations within the hypervariable herpes simplex virus (HSV) gene thymidine kinase (TK) gene lead to acyclovir (ACV) resistance. To uncover the existence of latent ACV-resistant (ACVR) HSV-1, we determined the genetic and functional variability of the HSV-1 TK gene pool in paired trigeminal ganglia (TG) of 5 immunocompetent individuals. The latent virus pool consisted of a donor-specific HSV-1 quasispecies, including one major ACV-sensitive (ACVS) and multiple phylogenetic-related minor ACV⁵ and ACV⁸ TK variants. Contrary to minor variants, major TK variants were shared between paired TG. The data demonstrate the coexistence of phylogenetic-related ACVS and ACVR latent HSV-1 in human TG.

Herpes simplex virus type 1 (HSV-1) is a highly prevalent human pathogen that causes a variety of diseases, including the potentially sight-threatening ocular disease herpetic keratitis [1]. HSV-1 establishes a lifelong latent infection in sensory neurons that innervate the anatomic site of primary infection and reactivates intermittently to cause recurrent lesions [1]. The trigeminal ganglion (TG) is a major site of HSV-1 latency. Recurrent HSV-1 infections are successfully treated with the nucleoside analogue acyclovir (ACV), in part due to the prerequisite conversion of the prodrug ACV to ACV monophosphate (ACVmp) by HSV-1 thymidine kinase (TK) [2].

Compared with other HSV-1 genes, the TK gene is highly polymorphic, and mutations within specific locations of the gene result in a functionally deficient TK protein or altered substrate specificity leading to ACV resistance [2–4]. Despite the extensive use of ACV for >3 decades, the incidence of ACV resistance is relatively low in the general population (0.1%–0.7%) but is more common in the immunocompromised (4%–14%) and in corneas of patients with herpetic keratitis (6.4%) [5]. Although TK is nonessential for viral replication in vitro, reactivation of latent TK-deficient HSV-1 is impaired in mice [6]. An analogous reactivation deficiency is assumed in humans. By contrasting, we have recently shown that genetically identical ACVS HSV-1 strains can be reisolated years later from the same cornea of patients with recurrent herpetic keratitis, suggesting that ACVS HSV-1 establishes latency and reactivates from the innervating TG [7]. The aim of the current study was to determine the prevalence of ACVS HSV-1 in latently infected human TG. Therefore, we analyzed the genetic and functional variability of the latent HSV-1 TK gene pool in the left and right TG of 5 immunocompetent individuals.

MATERIALS AND METHODS

Clinical Specimens

The left and right TG from 5 immunocompetent individuals (median age, 82 years; range, 74–93 years), 2 female and 3 male, were obtained at autopsy with a median postmortem interval of 5:15 hours (range, 5:05 to 7:30 hours). All donors had a neurologic disease history affecting the central nervous system (mainly Alzheimer’s and Parkinson’s disease) and the cause of death was not related to herpesvirus infections. The TG specimens were collected by the Netherlands Brain Bank (Netherlands Institute for Neuroscience, Amsterdam) from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained. All study procedures were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

HSV-1 TK Sequence Analyses

The amplification of the whole HSV-1 TK gene (1128 bp) from DNA extracted from fragmented TG was performed as described elsewhere [3, 7]. Agarose gel–purified TK amplicons were ligated into the TOPO-TA cloning vector (Invitrogen) and

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used to transform DH5α bacteria (Invitrogen). Per transformation up to 48 recombinant bacteria colonies were picked to sequence the TK insert using primers, located at the 5' and 3' end of the vector's multiple cloning site, provided by the TOPO-TA cloning kit (Invitrogen). The TK sequences were aligned to the consensus TK sequence of the reference HSV-1 strain H129 (GenBank accession No. GU_734772). A maximum likelihood phylogenetic tree of TK-derived TK nucleotide sequences was estimated under the general time reversible model using PhyML 3.0 software. The HSV-1 TK sequences obtained were deposited in the GenBank database under accession numbers HQ667768–HQ667782 and HQ685966–HQ686038.

**HSV-1 TK Functional Assay**

Representative HSV-1 TK variants were cloned into the pcDNA3 expression vector (Invitrogen) and transfected into Cos-7 cells (American Tissue Culture Collection; CRL-1651) using the FuGene-6 reagent according to the manufacturer’s instruction (Roche). After 48 hours of incubation at 37°C, transfected cells were stained intracellularly with goat anti-serum direct against HSV-1 TK (sc-28037) and an isotype control (sc-2028), followed by allophycocyanin-conjugated donkey anti-goat serum (sc-3860) according to the manufacturer’s instructions (all Santa Cruz Biotechnology). Fluorescence was detected on a FACS Canto II and analyzed using FACS Diva software (BD Biosciences).

The surplus transfected cells were lysed by freeze-thawing. Cell lysates were incubated with 1 volume of 200 µmol/L ACV in phosphate-buffered saline (acycloguanosine; Sigma-Aldrich) at 37°C. After 0, 30, 60, and 120 minutes, proteins were precipitated by adding 4 volumes of methanol. ACV and ACVmp levels were measured in quintuple by applying matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-QqQ-MS/MS; AB Sciex) [8]. Analyses were carried out by selected reaction monitoring. The monitored ion transitions were m/z 226 → m/z 152 for ACV and m/z 306 → m/z 152 for ACVmp. Ratios of ACVmp to ACV were calculated to determine the ACV-converting activity of the in vitro-produced TK proteins in time. The ACVmp/ACV ratios were corrected for the concentration of TK protein. The ACV-converting activity of the selected HSV-1 TK minor variants was calculated by normalizing the corrected ratios to the activity of the ACV-sensitive (ACV+) cognate major variant, which was set at 100% at 120 minutes incubation. The prevalence of latent ACV-R HSV-1 was defined by determining the frequency of TK sequences that encode a TK protein unable to convert ACV to ACVmp.

**RESULTS**

The aim of the study was to determine the prevalence of ACV-R HSV-1 in human TG. Hereto, the genetic and functional variability of the latent HSV-1 TK gene pool in the left and right TG of 5 individuals was analyzed. In total, 81 of 447 TK DNA sequences (18.1%) obtained were unique, with a mean of 8.6 distinct TK variants (range, 4–12 variants) per TG (data not shown). About three-fourths of the TK sequences contained nonsynonymous mutations resulting in amino acid mutations of the HSV-1 TK protein. Each TG contained 1 major TK protein variant, on average 85% (range, 71%–96%) of all TK protein variants per TG, and a variable number of minor TK protein variants (mean, 5.7; range, 2–9 minor TK variants per TG) (Table 1). The major TK protein variant was always genetically identical between paired left and right TG but distinguishable between the TG donors. The minor TK variants, however, were genetically related to the donor-associated major TK variant but were not shared with the contralateral TG of the same donor. Phylogenetic analyses demonstrated that both the major and minor TK protein variants, irrespective of left or right TG origin, clustered into distinct TG donor-specific clades (Figure 1A).

TK mutations conferring ACV resistance are commonly localized to the adenosine triphosphate–binding (amino acids 51–63) and nucleoside-binding site (amino acids 168–176), 5 designated TK gene regions conserved among Herpesviridae (amino acids 55–66, 79–91, 162–178, 212–226, and 218–292), and a 7-Gs homopolymer repeat region located at nucleotides 430–436 [3, 4, 7]. The majority of the TG-derived HSV-1 TK variants had mutations outside these TK function-related regions and have been documented to confer an ACV<sup>S</sup> phenotype of the respective HSV-1 strain, for example, S23N, L42P, I138V, and G281Q (Table 1) [3, 4, 7, 9]. Notably, several ACV resistance-associated TK variations described elsewhere were identified, consisting of mutations leading to premature stop codons (eg, amino acids 312stop and 250stop) or single amino acid substitutions, such as C336Y and R51W (Table 1) [3, 4, 10, 11]. To confirm the predicted ACV<sup>S</sup> and ACV<sup>R</sup> phenotype of the TG-derived HSV-1 TK protein variants, we developed a novel TK functional assay. The assay involves longitudinal mass spectrometric measurements of ACV and ACVmp concentrations in reaction mixtures containing ACV and an in vitro–produced TK protein variant (Figure 1B).

The ACV-converting activity of all 5 major TG-derived TK variants was comparable to that of TK derived from the ACV<sup>S</sup> HSV-1 reference strain KOS (data not shown). Among the minor TK variants, several variants had amino acid mutations suggesting an ACV<sup>R</sup> phenotype of the respective latent HSV-1 strain [3, 4, 10, 11]. Indeed, except for the A294T variant, all suspected TK amino acid mutations resulted in deficient or (in the case of L68P) impaired TK activity (Figure 1C).

**DISCUSSION**

The sequence and functional variability of the HSV-1 TK pool in latently infected TG specimens of 5 immunocompetent
Table 1. Herpes Simplex Virus Type 1 (HSV-1) Thymidine Kinase (TK) Variants Detected in Human Trigeminal Ganglia (TG)

<table>
<thead>
<tr>
<th>TG</th>
<th>Amino Acid Changes in TK Variant (% With Variant in Corresponding TG Specimen)</th>
</tr>
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<tbody>
<tr>
<td>TG1 left</td>
<td>Major variant: D14G, I138V, G240E (85)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: A17V (2.5); R30C (2.5); A118E (5); P209S (2.5); C336Y [4] (2.5)</td>
</tr>
<tr>
<td>TG1 right</td>
<td>Major variant: D14G, I138V, G240E (85.4)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: S23T (2.1); T122A (2.1); E146V (2.1); R236H (2.1); D258E (2.1); E371D (4.2)</td>
</tr>
<tr>
<td>TG2 left</td>
<td>Major variant: I138V (71.1)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: G21D (2.6); G129D [3] (5.2); G200D (2.6); A230V (2.6); P280Q (2.6); G302D (2.6); 312Stop (2.6); M322V (2.6)</td>
</tr>
<tr>
<td>TG2 right</td>
<td>Major variant: I138V (85)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: I54V (2.5); L169P (2.5); A266V (2.5); 281Stop [3] (2.5); P300T (2.5); R366H (2.5)</td>
</tr>
<tr>
<td>TG3 left</td>
<td>Major variant: R18C, I138V (96.8)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: L288S (2.1); A334T (2.1)</td>
</tr>
<tr>
<td>TG3 right</td>
<td>Major variant: R18C, I138V (93.5)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: A207T (2.2); D313G (2.2); L319P (2.2)</td>
</tr>
<tr>
<td>TG4 left</td>
<td>Major variant: S23N, E36K, O89R, I138V, G240E, R281Q (80.9)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: R26H (2.1); R51W [11] (2.1); G115R (4.2); L193P (2.1); G200D (2.1); A207D (2.1); G271D (2.1); A316T (2.1)</td>
</tr>
<tr>
<td>TG4 right</td>
<td>Major variant: S23N, E36K, O89R, I138V, G240E, R281Q (80.9)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: A114V (2.1); V119L (2.1); S123C (2.1); FS146-182Stop [3] (4.2); L178H (2.1); G185R (2.1); Q250L (2.1); M322I (2.1)</td>
</tr>
<tr>
<td>TG5 left</td>
<td>Major variant: L42P, I138V, G240E (87)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: R30C (2.2); L68P (2.2); R106H (2.2); A152V (2.2); L193I (2.2); A294T (2.2)</td>
</tr>
<tr>
<td>TG5 right</td>
<td>Major variant: L42P, I138V, G240E (87.2)</td>
</tr>
<tr>
<td></td>
<td>Minor variants: 250Stop (2.1); R256W (10) (2.1); D258E (2.1); L304P (4.2)</td>
</tr>
</tbody>
</table>

For major variants, the amino acid changes listed are different from the HSV-1 TK reference sequence (GenBank accession No. GU_734772). Minor variants represent sequences that contain the amino acid changes of the major variant in addition to the listed changes. Parenthetical values represent percentage of colonies per sample with the respective amino acid change. Underlined amino acid changes are known not to affect acyclovir sensitivity of the respective HSV-1 strain [3, 4, 7, 9] (based on Figure 1). Boldface changes are published acyclovir resistance-associated amino acid mutations [3, 4, 10, 11] (based on Figure 1C). (Reference citations are given in brackets.)

Abbreviations: FS, frameshift; Stop, premature stop codon at the indicated amino acid position.

individuals was assayed to determine the prevalence of latent ACVR HSV-1. Analogous to mucocutaneous HSV-1 infections, the latent HSV-1 pool is a mixed virus population. About 85% of the TK sequences in each TG represented one single major ACVS TK variant, identical between paired TG but distinguishable between TG donors. In addition, all TG contained multiple minor TK variants, both ACVS and ACVR TK variants, which were phylogenetically related to the cognate major TK variant.

In addition to the causative role of TK mutations in ACV resistance, the hypervariability of the TK gene provides insight into the clonal composition of an HSV-1 isolate [12]. In each TG donor, the latent virus pool consisted of a donor-specific HSV-1 quasi-species, of which only the major TK variant was detected in the paired left and right TG. This, along with the position of the major TK variant at the trunk of each donor-specific clade in the phylogenetic tree, suggests that the minor TK variants are derived from this major TK variant. The data strengthen the dogma that a single HSV-1 strain colonizes the TG and persists as a perennial source of latent virus and do not support TG superinfection with another virus strain during life [13, 14]. Colonization of both TG with the major variant may have occurred during primary infection or alternatively by autoinoculation of the contralateral anatomic site during recurrent infection. In contrast to the major TK variants, the phylogenetically related minor TK variants were not shared with the contralateral TG, suggesting that the minor variants are the result of intrahost virus evolution. Given their discordant presence between the paired TG, we postulate that the minor variants have evolved from the major variants within the respective TG, potentially during reactivation, and, less likely, within the TG-innervating mucosa during recurrent infection.

Although all major TK variants were ACVS, several minor TK variants had amino acid mutations suspected of having an ACVR phenotype of the cognate HSV-1 strain. We developed a novel TK functional assay and demonstrated that these TK variants were unable to convert ACV to ACVmp, which implies that the respective latent HSV-1 was ACVR. The prevalence of latent ACVR HSV-1 was estimated at 2.8%. Because we do not have information on the donors’ ACV treatment history, the ACVR variants detected could be due to the selective pressure of ACV treatment. Alternatively, the host immune system or other currently unknown selective factors...
of host or virus origin may account for the uncommonly high polymorphism of the HSV-1 UL23 TK gene resulting in both ACV resistance–associated and natural mutations of the progeny virus during the donor’s life. The presence of ACVR TK mutants in ganglia has been described elsewhere in a case report on an 18-year-old with a severe congenital immunodeficiency disorder [15]. The patient suffered from multiple episodes of severe HSV-1 infection, starting at 17 months of age and was extensively treated with ACV. The frequency of TK mutants suspected to be ACVR (25%) was much higher than that reported here and was most likely attributable to the patient’s deprived immune status and the continuing ACV selection pressure resulting in the emergence and persistence of ACVR viruses early in life [15]. In contrast, the TG donors analyzed here did not have a history of immunodeficiency or severe HSV-1 infections, reflecting the presence of latent
ACV<sup>R</sup> HSV-1 in the general population. Latent ACV<sup>R</sup> virus may reactivate by itself from human TG, or functional TK from a coreactivating latent ACV<sup>S</sup> virus could complement the reactivation deficiency of the ACV<sup>R</sup> virus in trans to spread to the innervating mucosa, leading to recurrent disease [16, 17]. The coexistence of both ACV<sup>R</sup> and ACV<sup>S</sup> phylogenetically related HSV-1 in individual neurons within the same human ganglion supports the latter option [15].

In conclusion, we report on the presence of phylogenetically related ACV<sup>S</sup> and ACV<sup>R</sup> HSV-1 in latently infected TG of immunocompetent individuals. Reactivation of latent ACV<sup>R</sup> HSV-1 from its ganglionic stronghold poses a risk of developing ACV refractory clinical disease in immunodeficient patients or at immunoprivileged anatomic sites, such as the cornea [7].

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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