Acyclovir-Resistant Corneal HSV-1 Isolates from Patients with Herpetic Keratitis

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The prevalence and molecular characteristics of isolates from 173 immunocompetent patients with herpetic keratitis (HK) who were infected with acyclovir (ACV)–resistant (ACVR) corneal herpes simplex virus (HSV)–1 was determined. Isolates from 11 (6.4%) of the patients were ACVR, and 9 of these 11 patients were refractory to therapy with ACV; the ACVR isolates from 5 and 5 of these 9 patients were cross-resistant to ganciclovir and foscarnet, respectively. Of the 11 ACVR isolates, 10 had, in the thymidine kinase gene, mutations that presumably conferred the ACVR phenotype. These data demonstrate a relatively high prevalence of corneal HSV-1 ACVR isolates in patients with HK, which emphasizes the need to monitor for ACV susceptibility in patients with HK who are refractory to therapy with ACV.

HSV-1 is a leading cause of corneal disease and blindness in humans, largely because of its recurrent nature [1]. Herpetic keratitis (HK) manifests predominantly as either infectious epithelial keratitis (IEK) or herpetic stromal keratitis (HSK). The drugs of choice for treatment of HK are nucleoside analogues, such as acyclovir (ACV) and ganciclovir (GCV), and, less frequently, the viral DNA–synthesis inhibitors foscarnet (FOS) and cidofovir [2]. These treatments, along with corticosteroids in the case of HSK, have resulted in a significant reduction of HSV-1–induced corneal blindness [1, 2]. The antiviral activity and selectivity of these compounds are different: whereas ACV and GCV depend on metabolic activation by HSV-encoded thymidine kinase (TK), FOS inhibits viral DNA polymerase directly, as a substrate analogue of pyrophosphate formed during DNA synthesis [3]. Among immunocompromised patients with HSV-1 disease, the prevalence of ACV-resistant (ACVR) HSV-1 isolates is much higher (4.3%–14%) than that in immunocompetent patients (0.1%–0.6%) [4–6]. This difference is most likely due to longer mucosal persistence of ACVR HSV variants, which, in turn, is due to impaired local immune responses [1]. In ~95% of cases, ACV resistance is associated with alterations within the HSV-1 TK gene. Less frequent are mutations in the viral DNA polymerase gene that may lead to cross-resistance to FOS [7, 8]. In contrast to the extensive study of genital herpes and herpes labialis, large surveys of the incidence of ACV resistance in patients with HK are lacking [4–8]; thus far, there have been only a few anecdotal case reports of ACVR corneal HSV-1 in patients with HK [4, 9–12]. In the present study, we determine the prevalence and molecular characteristics of ACVR corneal HSV-1 in a cohort of 173 patients with HK; in addition, cross-resistance to GCV and FOS is determined.

Patients, materials, and methods. At the Rotterdam Eye Hospital, between 1981 and 2005, corneal swabs from 173 patients who had suspected herpetic corneal lesions but who were otherwise healthy were obtained for diagnostic purposes. Virus was grown and typed for HSV-1, as described elsewhere [13]. The classification of HK was based on clinical criteria [1]. The study was performed according to the tenets of the Declaration of Helsinki and was approved by the local ethical committee, and written informed consent was obtained from the patients.

The susceptibility of the corneal HSV-1 isolates to ACV (GlaxoSmithKline), GCV (Roche), and FOS (AstraZeneca) was determined by real-time PCR (qPCR) assay, as described elsewhere [14]. A plaque-reduction assay (PRA) was employed to confirm the results of qPCR [14]. In brief, Vero monolayers in 24-well culture plates were inoculated with 50 plaque-forming units of HSV-1. After incubation for 1 h at 37°C, the viral inoculum was removed and the cells were incubated, in triplicate, with different concentrations of ACV, GCV, or FOS that were diluted in Dulbecco’s minimal essential medium supplemented with 1% fetal bovine serum and 0.6% methylcellulose (Acros Organics). At 72 h after inoculation, plaques were visualized and counted.

qPCR was performed in a manner similar to that used for PRA, except that the cells were infected with 100-fold–diluted stocks of corneal HSV-1 isolate [14]. At 24 h after inoculation,
Table 1. Characteristics of patients with herpetic keratitis and corneal herpes simplex virus (HSV)-1 isolates resistant to ACV.

<table>
<thead>
<tr>
<th>Patient (age; years; sex)</th>
<th>IC_{50} µmol/L</th>
<th>ACV</th>
<th>GCV</th>
<th>FOS</th>
<th>Mutation in TK</th>
<th>Clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRA</td>
<td>qPCR</td>
<td>PRA</td>
<td>qPCR</td>
<td>PRA</td>
<td>At culture date</td>
</tr>
<tr>
<td>R1 (40; F)</td>
<td>9.5</td>
<td>4.4</td>
<td>0.2</td>
<td>0.4</td>
<td>47</td>
<td>L178R</td>
</tr>
<tr>
<td>R2 (78; F)</td>
<td>2.8</td>
<td>1.5</td>
<td>1.3</td>
<td>0.2</td>
<td>101</td>
<td>T345P</td>
</tr>
<tr>
<td>R3 (69; M)</td>
<td>5.8</td>
<td>3.5</td>
<td>3.2</td>
<td>3.9</td>
<td>241</td>
<td>None</td>
</tr>
<tr>
<td>R4 (72; M)</td>
<td>&gt;50</td>
<td>45.5</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>40</td>
<td>del36E</td>
</tr>
<tr>
<td>R5 (52; M)</td>
<td>21.5</td>
<td>3.2</td>
<td>1.8</td>
<td>1.1</td>
<td>46</td>
<td>S181N</td>
</tr>
<tr>
<td>R6 (67; M)</td>
<td>35.4</td>
<td>13.5</td>
<td>27.4</td>
<td>29.1</td>
<td>81</td>
<td>del194I</td>
</tr>
<tr>
<td>R7 (29; M)</td>
<td>16.1</td>
<td>4.5</td>
<td>1.6</td>
<td>0.7</td>
<td>78</td>
<td>L178R</td>
</tr>
<tr>
<td>R8 (51; M)</td>
<td>12.6</td>
<td>7.6</td>
<td>13.3</td>
<td>3.1</td>
<td>54</td>
<td>Frameshift 146</td>
</tr>
<tr>
<td>R9 (69; M)</td>
<td>10.6</td>
<td>4.3</td>
<td>14.3</td>
<td>7.4</td>
<td>54</td>
<td>Frameshift 146</td>
</tr>
<tr>
<td>R10 (79; M)</td>
<td>2.4</td>
<td>1.4</td>
<td>0.2</td>
<td>0.2</td>
<td>41</td>
<td>I78S</td>
</tr>
<tr>
<td>R11 (33; F)</td>
<td>3.2</td>
<td>1.3</td>
<td>0.2</td>
<td>0.6</td>
<td>105</td>
<td>D258N</td>
</tr>
</tbody>
</table>

NOTE. ACV, acyclovir; FOS, foscarnet; GCV, gancyclovir; IEK, infectious epithelial keratitis; ISK, infectious stromal keratitis; KU, keratouveitis; NSK, necrotizing stromal keratitis; PRA, plaque-reduction assay; qPCR, real-time polymerase chain reaction; TK, HSV gene for thymidine kinase.

a Cutoff values for both resistance to ACV and resistance to GCV are >1 µmol/L in qPCR and >2 µmol/L in PRA, and cutoff values for resistance to FOS are >200 µmol/L; strains that are cross-resistant to either GCV or FOS are underlined. For qPCR, the mean ± SD values for isolates sensitive to ACV and for the isolates sensitive to GCV were 0.2 ± 0.1 µmol/L (range 0.1–0.6 µmol/L) and 0.2 ± 0.3 µmol/L (range 0.1–1.1), respectively; for PRA, the mean ± SD values for isolates sensitive to ACV, isolates sensitive to GCV, and isolates sensitive to FOS were 0.3 ± 0.3 µmol/L (range, 0.1–1.9 µmol/L), 0.3 ± 0.4 µmol/L (range, 0.2–1.8 µmol/L), and 50 ± 18 (range, 10–91) µmol/L, respectively.

b The TK gene sequence of each isolate was compared with that of ACV-susceptible HSV-1 reference strain KOS (GenBank accession number GI:125436); amino acid substitutions, deletions (del), or frameshift mutations potentially conferring resistance to ACV are shown. The GenBank accession numbers of isolates from patients R01–R11 are EU541360, EU541361, EU541362, EU541363, EU541364, EU541365, EU541366, EU541368, EU541367, and EU541370, respectively.

the supernatant was discarded and the cells were treated with 300 µL of lysis buffer (2.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl, 0.45% Tween 20, 0.45% Nonidet-P40, and 200 µg/mL protease K) and were incubated for 90 min at 56°C. A 10-µL portion of the 10-fold diluted lysate was subjected to qPCR by use of an Applied Biosystems 7000 Sequence Detection system, as described elsewhere [13]. Viral load was determined on the basis of a standard curve generated on a stock of HSV-1 strain McIntyre counted by electron microscopy (Advanced Biotechnologies) [13], and the HSV-1 isolates were assayed at least 3 times. The IC₅₀ was defined as the concentration of antiviral drug that reduced the number of plaques or viral copies by 50%, compared with what was observed for control infected cells to which antiviral drug had not been added. Isolates were considered to be resistant to ACV and GCV if the PRA-based IC₅₀ was >2 µmol/L and the qPCR-based IC₅₀ was >1 µmol/L; isolates were considered to be resistant to FOS if the PRA-based IC₅₀ was >200 µmol/L. [14].

DNA was isolated from supernatants of HSV-1–infected Vero cells by use of a MagNA Pure LC total nucleic acid kit (Roche). The TK gene was amplified by PCR with 200 pmol of forward (5’-TGCGCTGAACTCCGGCACA-3’) and reverse (5’-CCCATAACGCGCAGATGC-3’) primer. Amplification conditions included denaturation for 4 min at 94°C; 30 cycles of 1 min at 94°C, 45 s at 50°C, and 2 min at 72°C; and a final extension step for 5 min at 72°C. The 1131-bp amplicon was purified by use of a MINelute gel-extraction kit (QIAGEN). The entire open reading frame of TK was sequenced by use of an ABPrism 3100 analyzer and BigDye Terminator cycle sequencing kit (Applied Biosystems), by use of both of the external primers (as defined above) and both sense (5’-CGCCAGATAGATGCGC-3’) and antisense (5’-TCTGCTTATTTATGCGTCAT-3’) internal primers.

The size of the TK protein was determined by Western blotting using 2 polyclonal antibodies (sc-28037 and sc-28038; Santa Cruz Biotechnology) directed against different regions of HSV-1 TK. Extracts from HSV-1–infected Vero cells were prepared by lysis and denaturation for 10 min at 95°C and then were subjected to electrophoresis through an acrylamide gel and were blotted onto a polyvinylidene fluoride membrane (Amersham). After overnight blocking with 5% milk powder, the membrane was incubated with primary (1:500) and secondary (1:5000; horseradish peroxidase–conjugated donkey anti-goat; Santa Cruz Biotechnology) antibodies. Subsequently, chemiluminescent substrate (Amer-
sham) was applied to the membrane for 1 min before the latter was exposed to x-ray film, according to the manufacturer’s instructions.

**Results.** The cohort of patients with HK consisted of 102 men and 71 women (mean age, 50±22 years), whose clinical diagnoses included IEK (n=79), HSK (n=85), and kerato-uveitis (n=9). All patients had been treated with ACV during the year before isolates were obtained. At the time when the isolates were obtained, 25 patients were being treated with ACV and 4 patients were being treated with trifluorothymidine. qPCR analyses revealed that 11 (6.4%) of the 173 isolates were ACVR, and this was confirmed by subsequent PRA assays (table 1). Of these 11 ACVR isolates, 5 (from patients R3, R4, R6, R8, and R9) were resistant to GCV, and 1 isolate (R3) was cross-resistant to both GCV and FOS (table 1). The results of both the PRA-based and qPCR-based IC50 were reproducible and correlated significantly, for both ACV (r=0.82; P=.02) and GCV (r=0.94; P<.0001, by Spearman’s rank-correlation test). Of the 11 ACVR isolates, 4 (36.4%) were from patients (specifically, R1, R2, R5, R7, and R9) who were being treated with topical ACV at the time when the isolates were obtained, a proportion that is significantly greater than that for ACV-sensitive (ACVS) patients (21 [13.0%] of 162) (P<.01, by χ² test). Of the 11 patients who were ACVR, 9 (patients R1–R9) were clinically refractory to treatment with ACV (e.g., there was slow epithelial healing or development of severe HSK). Of these 9 patients, 5 (patients R1, R2, R5, R7, and R9) were switched to topical FOS therapy and 2 (patients R6 and R8) were switched to topical GCV therapy. Initially, this change was beneficial to all patients; however, during the >3-year-long follow-up period, corneal blindness developed in 7 (64%) of the 11 patients who were ACVR (specifically, patients R2-R5 and R7–9), compared with only 11 (6.8%) of 162 patients who were ACVS (P=.01, by χ² test) (table 1).

Because ~95% of the mutations causing ACVR occur in the TK gene [4, 7, 8], the entire TK gene of the 11 ACVR isolates was sequenced. Alignment with both ACVR (n=74) and ACVS (n=40) HSV-1 TK sequences available at GenBank revealed numerous amino acid mutations in all ACVR isolates (table 1) [7, 8; and data not shown]. Of the 11 ACVR isolates, 10 had TK-gene mutations that potentially conferred the ACVR phenotype; 2 isolates (from patients R1 and R7) had an amino acid substitution at residue 178 (specifically, Leu178Arg) [7, 8]. The isolate from patient R4 had a 3-nt in-frame deletion at position 36, leading to loss of glutamic acid, and the isolate from patient R6 had a 3-nt in-frame deletion at position 194, leading to loss of isoleucine. Four amino acid substitutions, not documented as being from ACVS HSV-1 isolates, were found: isoleucine→serine (residue 78; patient R10), serine→asparagine (residue 181; patient R5), aspartic acid→asparagine (residue 258; patient R11) and threonine→proline (residue 354; patient R2). The

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**Figure 1.** Western blot analysis of altered herpes simplex virus type 1 (HSV-1)–encoded thymidine kinase (TK) protein in acyclovir (ACV)–resistant corneal HSV-1 isolates. The results shown are for ACV-resistant corneal HSV-1 isolates from patients R8 (lane 3) and R9 (lane 4), for the ACV-sensitive HSV-1 reference strain KOS (GenBank accession number GI:125436) (lane 1), and for a mock-infected negative control (lane 2). The anti–HSV-1 TK polyclonal antibodies used were directed toward either the N-terminal (sc-28037) or the internal TK region (sc-28038), located between residues 50 and 100 and between residues 215 and 265, respectively (Santa Cruz Biotechnology sales manager, personal communication). As is indicated by the asterisks (*), no signal is seen with the internal antibody, because the frameshift mutation alters the residue composition after residue 146, an alteration that is not recognized by the internal antibody. The locations of both the wild-type (black arrow) and truncated (white arrow) TK proteins are also indicated. Ab, antibody; kd, kilodalton; MW, molecular weight.
isolates from patients R8 and R9 had an identical 1-nt insertion in the G-repeat region, located between residues 430 and 437 [7, 8], leading to a frameshift and premature stop codon at residue 208 (table 1). The remaining amino acid mutations have been found in both ACVs and ACVR strains and therefore are unlikely to be associated with resistance to ACV [4, 7, 8].

Western blotting confirmed the predicted truncation of the TK protein of the isolates from patients R8 and R9 (figure 1). The truncated TK protein was detected by the N-terminal antibody, whereas no protein was detected by the internal antibody; this N-terminal antibody binds at residues 215–216, a location that is C-terminal to the frameshift location. The remaining amino acid mutations identified in the ACVR isolates were also observed in ACVs isolates and therefore can be presumed to represent natural polymorphisms [2; and data not shown].

Discussion. Since the discovery of ACV 30 years ago, the remarkable efficacy and safety of this drug has been a milestone in the treatment of HSV infections [3, 4]. Until now, resistance to ACV had rarely been reported in otherwise healthy patients [4–6]. The present study is the first to report an unprecedented relatively high (6.4%) prevalence of ACVR corneal HSV-1 isolates in immunocompetent patients with HK. We hypothesize that this high prevalence could be due to the unique immune-privileged site of the cornea, which differs from other HSV-1 infection sites [15]. At the Rotterdam Eye Hospital, where all patients with HSV-1 have been treated, prophylactic treatment with ACV has greatly increased since 1994; because all ACVR isolates identified in the present study came from patients who presented after 1994, we hypothesize that the increase in ACV prescriptions may have influenced the emergence of ACVR corneal HSV-1 at this hospital.

Several mutations in the HSV-1 TK gene were detected that may have conferred the ACV resistance seen in 10 of the 11 corneal isolates that were ACVR. These mutations have not been described in ACVs strains and include multiple amino acid deletions and mutations [7, 8; and data not shown]. The arginine-leucine substitution at residue 178 in the isolates from patients R1 and R7 is of particular interest because it is located within the nucleoside-binding site of TK [7, 8]. We also report a G insertion at nucleotide position 430 in 2 ACVR isolates (from patients R8 and R9), with a resulting frameshift leading to a truncated TK protein (figure 1). The GCV/FOS–cross-resistant ACVR isolate from patient R3 had no obvious mutations in the TK gene, suggesting that mutations within the DNA polymerase gene are involved [7, 8]. Of the 5 ACV/GCV–cross-resistant isolates, 4 (from patients R4, R6, R8, and R9) had frameshift mutations and deletions in the TK gene. In comparison, the mutations in the TK gene identified in the ACV/GCV–cross-resistant corneal HSV-1 isolates are probably more detrimental to the function of the TK protein to convert both ACV and GCV into active compounds. Although the molecular and functional data suggest that these newly identified TK mutations are significant, no definitive conclusion regarding them and their role in ACV resistance can be drawn without confirmatory testing using site-directed mutagenesis.

In vitro resistance assays and molecular characterization of HSV-1 isolates are strong diagnostic tools and should be implemented to rationalize a switch in antiviral therapy for patients refractory to treatment [3, 4]. In the cohort monitored in the present study, 9 of 11 patients with ACVR HSV-1 were clinically resistant to ACV therapy, and 7 of these 9 were eventually switched to other antiviral drugs. Whereas all patients responded initially, corneal blindness eventually developed in 7 of the 11 patients with ACVR HSV-1. Because the majority of these patients had HSK at the start of the present study (patients R2 and R4–R8; table 1), a chronic T cell–mediated inflammatory corneal disease, their ACVR phenotype may be only partly responsible for the long-term deterioration of the corneal tissue caused by this disease [1]. Nonetheless, if the antiviral–resistance data had been available beforehand, a switch to an alternative antiviral drug could have been initiated to lower the corneal load, potentially limiting both virus-mediated cytopathology and intracorneal deposition of viral antigens recognized by the pathogenic intracorneal T cell response [1].

In conclusion, the data provided by the present study show a relatively high prevalence of resistance to ACV in patients with HK, compared with that in immunocompetent individuals with other HSV-1–related diseases [4–6]. Given the high prevalence and severity of HK, we suggest that determination of the antiviral–resistance patterns of corneal HSV-1 isolates from HK patients who are refractory to antiviral therapy should be implemented as a standard diagnostic tool. This immediate in vitro testing can be used to rationalize a switch to treatment with GCV, trifluridine and vidarabine, or more-toxic antiviral drugs, such as FOS or cidofovir. Such a switch in treatment will save precious therapeutic time and ultimately may prevent development of severe disease, including corneal blindness.

References


