Development of Herpes Simplex Virus Disease in Patients Who Are Receiving Cidofovir

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Background. Cidofovir is a nucleotide analogue with antiviral activity against a wide range of DNA viruses, including herpes simplex virus (HSV). In vitro resistance to cidofovir has been reported with use of laboratory HSV strains; clinical failure of cidofovir therapy for HSV disease has also been reported with an isolate that was susceptible by in vitro testing.

Patients and methods. Three patients with HSV disease that was unresponsive to cidofovir therapy had viral isolates obtained and stored; the isolates were analyzed for antiviral susceptibility by an antigen reduction assay (ARA). PCR cloning and automated sequencing were performed for isolates that displayed in vitro resistance. Mutations were identified by comparison with the appropriate HSV consensus sequence.

Results. An HSV type 2 (HSV-2) isolate recovered from patient 2 displayed in vitro cidofovir resistance with an inhibitory concentration of 50% (IC50) of 13.06 μg/mL. HSV type 1 isolates recovered from patients 1 and 3 had elevated IC50s to cidofovir (7.32 and 8.23 μg/mL, respectively); however, these isolates did not meet the cutoff point for resistance according to the ARA. Sequencing of a cidofovir-resistant HSV-2 isolate revealed several DNA polymerase mutations that had not been previously described during in vitro resistance selection.

Conclusions. To our knowledge, this is the first report of cidofovir resistance in HSV developing in vivo. The sequenced clones all contained mutations truncating the pol C′ end, suggesting that this region may be critical for cidofovir antiviral activity. In addition, the presence of multiple mutations suggests that the altered DNA polymerase of cidofovir-resistant virus may have introduced additional mutations into the viral genome. Introduction of the mutations identified in wild-type HSV strains is needed before the resistance phenotype can be definitively associated with any of the mutations found. Additional studies are needed to delineate the mechanisms of cidofovir resistance in HSV.

Herpes simplex virus (HSV) types 1 and 2 (HSV-1 and HSV-2, respectively) are closely related DNA viruses that can cause mucocutaneous and disseminated disease in both immunocompetent and immunocompromised patients. Reactivation of HSV disease is a particular problem in patients who have undergone allogeneic bone marrow transplantation, and therefore, prophylaxis with acyclovir is administered to such patients [1].

Acyclovir is used as first-line therapy for HSV disease; however, resistance to acyclovir can develop and is often associated with clinical failure. Several recent studies have highlighted the increasingly recognized problem of acyclovir-resistant HSV in allogeneic bone marrow transplant recipients [2, 3]. Factors associated with the development of acyclovir resistance include prior treatment with acyclovir, HIV-1 infection, and graft-versus-host disease (GVHD) [2–4].

Resistance to acyclovir most often occurs through mutations in the viral thymidine kinase (TK) required for phosphorylation of acyclovir [5]. Other anti-herpes virus medications, such as foscarnet and cidofovir, retain their activity against TK-deficient strains of HSV. Mutations in the HSV DNA polymerase occur less frequently but may confer resistance to either foscarnet or acyclovir [6]. Foscarnet is considered the treatment of choice for acyclovir-resistant HSV infection [7]. Infections with HSV isolates that are resistant to both acyclovir and foscarnet have limited treatment options, one of which is cidofovir.
Cidofovir is a nucleotide analogue that is active against a wide range of DNA viruses, including HSV [8, 9]. Importantly, cidofovir does not depend on viral TK for activation and retains its activity against TK-deficient strains of HSV in vitro [5, 8]. Cidofovir has been shown to be highly active in mouse models of HSV infection, including both wild-type and TK-deficient strains of HSV [8]. Clinically, cidofovir has been used successfully for treatment of HSV infection, including infection with strains in vitro and clinical resistance to both acyclovir and foscarnet [10, 11].

We describe 3 bone marrow transplant recipients with HSV disease that developed or progressed despite receipt of cidofovir therapy. In 1 of these cases, cidofovir resistance was confirmed with an antigen reduction assay, and candidate genotypic correlates were identified by sequencing the isolate.

CASE REPORTS

**Patient 1.** A 46-year-old white man underwent autologous bone marrow transplantation for non-Hodgkin lymphoma in 1997. He subsequently developed myelodysplastic syndrome and underwent a nonmyeloablative allogeneic bone marrow transplantation 6 months before his presentation to the hospital with severe GVHD of the skin and gastrointestinal tract. Four weeks before presentation, the patient stopped taking all immunosuppressive and prophylactic medications, including oral acyclovir. The patient was treated with high-dose corticosteroids and tacrolimus, as well as prophylactic medications consisting of levofloxacin, fluconazole, aerosolized pentamidine, and acyclovir (5 mg/kg iv q12h). Symptoms of GVHD persisted, and mycophenolate mofetil was added to the treatment regimen.

On day 19 of hospitalization, the patient developed gross hematuria, and the result of adenovirus PCR of a urine specimen was positive. Treatment with cidofovir was started at a dosage of 5 mg/kg intravenously once per week, and acyclovir prophylaxis was discontinued. Probenecid was administered in a dosage of 2 g orally 3 h before and 1 g 2 and 8 h after the cidofovir infusion. The patient received 2 doses at 1-week intervals (the induction phase) and then started receiving maintenance doses of cidofovir (5 mg/kg) every 2 weeks. Approximately 3 weeks into cidofovir therapy, the patient developed dysphagia and gastrointestinal bleeding. A tongue ulcer was noted the day after the bleeding began, and treatment with acyclovir (10 mg/kg iv q8h) was initiated. Esophagogastroduodenoscopy and colonoscopy were performed, and immuno histochemical stains and cultures of biopsy specimens were positive for HSV-1.

The patient had minimal response to acyclovir therapy, and subsequent sensitivity testing revealed that the HSV isolate had elevated IC50s of acyclovir (IC50, 35.8 μg/mL) and cidofovir (IC50, 7.32 μg/mL); the foscarnet IC50 was 8.10 μg/mL. Cidofovir and acyclovir therapy was discontinued, steroid doses were tapered, and therapy with foscarnet (90 mg/kg iv q12h) was initiated. The patient had gradual improvement and was discharged from the hospital ~3 weeks after beginning foscarnet therapy.

**Patient 2.** A 43-year-old man with AIDS and a history of cytomegalovirus (CMV) retinitis received a diagnosis of Burkitt lymphoma and began undergoing chemotherapy. Before commencement of chemotherapy, the patient started receiving treatment with cidofovir (with probenecid; 5 mg/kg iv every 2 weeks) for CMV infection prophylaxis. Ganciclovir was not used because of concerns about additional bone marrow suppression after chemotherapy. During his second cycle of chemotherapy, the patient developed a vesicular, pruritic eruption on his right buttock and penis. One of the lesions was unroofed, and samples tested positive for HSV-2 by PCR. At this point, the patient had received a total of 6 doses of cidofovir (5 mg/kg) at 2-week intervals, with the last dose having been administered 6 days before the development of the lesions. The patient started receiving acyclovir (400 mg po q.i.d.), and the lesions resolved. Subsequent sensitivity testing of the isolate revealed cidofovir resistance, with a significantly elevated cidofovir IC50 (13.06 μg/mL).

**Patient 3.** A 48-year-old white woman with chronic lymphocytic leukemia underwent a 5/6 matched cord blood transplantation after receipt of a preparative regimen, which consisted of total-body irradiation, melphalan, and antithymocyte globulin. Her course was complicated by severe mucositis, which began 3 days prior to transplantation. Her prophylactic medications consisted of levofloxacin, fluconazole, FK-463 (micafungin), cyclosporin, prednisone, and acyclovir (5 mg/kg iv q12h). Mucositis persisted and her acyclovir dose was increased to 10 mg/kg intravenously every 8 h.

Despite receipt of intravenous acyclovir, the patient’s mucositis worsened, and she developed bilateral pulmonary infiltrates associated with respiratory failure that required mechanical ventilation on day 30 after transplantation. Cultures of the oral lesions were persistently positive for HSV-1, as were cultures of bronchoalveolar lavage fluid.

Susceptibility testing of the initial isolate showed elevated IC50s of both acyclovir (IC50, 4.36 μg/mL) and foscarnet (IC50, 134.13 μg/mL). Acyclovir therapy was discontinued, and cidofovir therapy (with probenecid) was instituted (5 mg/kg iv every week). Despite administration of therapy with cidofovir, the patient’s clinical condition deteriorated, and cultures remained positive for HSV. Susceptibility testing was repeated for HSV recovered from a culture performed 3 weeks into cidofovir therapy, and the HSV showed an increased IC50 of cidofovir (8.23 μg/mL). Given the elevated IC50 and lack of clinical response, cidofovir therapy was discontinued, and intravenous...
ganciclovir therapy was started. The patient’s clinical status continued to decline, and she died.

Autopsy findings included diffuse, bilateral interstitial pneumonia with hemorrhage and esophagitis, both of which were consistent with severe disseminated HSV infection. Pneumocytes with intranuclear inclusions were visualized by light microscopy; however, the results of immunohistochemical stains for HSV, varicella-zoster virus, and CMV were negative. On the basis of culture data and the findings of pathologic examination, the final diagnosis was severe HSV pneumonitis with diffuse alveolar damage, resulting in cardiopulmonary failure.

**METHODS**

**Virus isolation.** Swab specimens from papules, vesicle base, or ulcers were collected into M4 transport medium (Micro Test) and inoculated into cultures of human embryonic lung fibroblasts (HELs). HSV isolates were typed with specific monoclonal antibodies (Diagnostic Product Co.)

**Antigen reduction assay.** HSV sensitivity was tested using an antigen reduction assay, as previously described [12]. HELF monolayers seeded in 96-well plates were inoculated with 10-fold dilutions (10⁻² to 10⁻⁴) of the clinical isolates. Antivirals were added to triplicate wells at the following final concentrations: cidofovir, 3, 30, and 30 μg/mL; acyclovir, 1, 3, and 9 μg/mL; and foscarnet, 13, 30, and 120 μg/mL. A strain of HSV-2 that was known to be antiviral susceptible was included as an assay control in each run. At 24 hours after infection, the plates were fixed with 0.05% glutaraldehyde, and uncoated sites were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and were incubated with rabbit anti-HSV IgG (Accurate) in PBS-BSA. Plates were washed with 0.1% Tween 20 in PBS, and bound antibodies were revealed with goat anti-rabbit IgG conjugated with horseradish peroxidase (Tago) and 2,2'-azino-bis(3-ethyl-benzothiazoline sulfonic acid (Sigma). The optical density was adjusted by subtracting the background, determined in uninfected wells, from each set of readings. The IC₅₀ was defined as the antiviral concentration that reduced the adjusted optical density to 50% of the reading in untreated viral wells. Cidofovir resistance was defined as an IC₅₀ of >8 μg/mL and of >2 times the IC₅₀ of the cidofovir-susceptible control, acyclovir resistance was defined as an IC₅₀ of >2.5 μg/mL and of >2 times the IC₅₀ of the acyclovir-susceptible control, and foscarnet resistance was defined as an IC₅₀ of >100 μg/mL and of >2 times the IC₅₀ of the foscarnet-susceptible control. Susceptibility thresholds were defined by the mean + 3 SDs of >20 HSV-1 and HSV-2 clinical isolates obtained from immunocompetent hosts before commencement of antiviral therapy.

**DNA sequencing.** The viral isolate from patient 2 was PCR-cloned and sequenced. DNA was extracted from a freeze-down of the viral isolate using the QiaAamp DNA Blood Mini Kit (Qiagen) and was directly subjected to PCR using the Advantage GC-2 PCR kit (BD Biosciences-Clontech), with primer sets directed at the HSV-2 DNA polymerase gene (Appendix, table A1, available online). Three independent PCR amplifications (using each set of primers) of the specimen were performed, and products from each of these reactions were cloned and sequenced. PCR products were cloned into plasmids using the TOPO TA Cloning Kit For Sequencing (Invitrogen). Sequences were obtained using M13 Forward and M13 Reverse primers, the BigDye Terminator v3.1 Cycle sequencing kit, and the ABI Prism 3100 Genetic Analyzer automated sequencing system.

**RESULTS**

The IC₅₀ values for patient samples are shown in the Appendix, table A2 (available online), and are depicted with drug administration and clinical events in figure 1. Susceptibility cutoff values were determined with an antigen reduction assay and by comparison with a susceptible HSV-2 control, as described in Methods.

The DNA polymerase of 3 clones obtained from the patient 2 isolate were sequenced. Each clone had multiple mutations, 5 of which were shared by all 3 clones (Appendix, table A3, available online). The shared mutations were in nonconserved areas of the HSV-2 DNA polymerase. In addition, each of the clones had a stop codon mutation that truncated the 3' end (C-terminus) of the polymerase gene.

**DISCUSSION**

To our knowledge, patient 2 represents the first report of HSV cidofovir resistance developing in vivo. HSV resistance to cidofovir has been selected for in the laboratory setting and was found to occur through mutations in the viral DNA polymerase in both conserved and variable regions of the polymerase [13–15]. Mendel et al. [13] first described in vitro selection of cidofovir resistance in HSV-2. Genotypic analysis of the laboratory derived isolate showed a glycine to serine mutation at position 506 of the DNA polymerase. Resistance to cidofovir in HSV-1 laboratory strains KOS and 17 has also been selected for in vitro [13, 14]. In the HSV-1 KOS strain, 3 mutations were found after selection with cidofovir: valine-573 to methionine (δ-region C), alanine-136 to threonine, and arginine-700 to methionine (region II) [13]. These resistant isolates remained susceptible to acyclovir and foscarnet in vitro. The R700M mutation was also found after in vitro selection with cidofovir in the HSV-1 laboratory strain 17 [15]. The authors also found several other cidofovir resistance mutations within conserved regions II, III, and VI (S724N, T821M, and L778M respectively) and outside of conserved regions of the DNA polymerase (I1028T).
gene, including 5 shared by all clones. None of the shared mutations were located within the conserved regions (I-VII or δ-region C) of the polymerase gene. Several mutations associated with cidofovir resistance in vitro have been located outside of the conserved regions; none of the mutations identified in patient 2 correspond to those described previously in non-conserved regions of the polymerase gene.

Because all 3 sequenced clones shared these 5 mutations, it seems likely that ≥1 of these mutations conferred cidofovir resistance on the isolate. Because cidofovir is a DNA polymerase inhibitor, cidofovir inhibition of DNA polymerase may have reduced polymerase fidelity, increasing the likelihood of mutations in the polymerase and other genes of viruses that were replicating in this individual.

The sequenced specimen was a viral isolate, grown in cell culture in HELF cells. Thus, this specimen contained replication-competent virus. In addition to the 5 shared mutations, each polymerase clone contained additional mutations not shared with the other clones, including mutations resulting in different stop codons that truncated the polymerase genes. One possible explanation for this large number of additional mutations is that the mutant (cidofovir resistance-conferring) HSV polymerase gene may have had considerably lower replicative fidelity (even in the absence of cidofovir) than did the wild-type polymerase gene, and that this mutant polymerase gene could have introduced additional mutations into these viral clones during replication in cell culture. If correct, this explanation suggests that the cidofovir-resistant virus would have significantly lower replication efficiency than would a wild-type virus, because a significant proportion of replicated virus genomes appeared to be defective. These mutations are unlikely to have been introduced by PCR amplification of the polymerase sequences, because the amplification system included a proofreading polymerase that reduces the frequency of such mutations. The role of any of these mutations in the resistance phenotype seen in our patient needs to be confirmed by marker transfer experiments introducing each mutation into wild-type HSV and repeating susceptibility testing. Only then can a direct correlation between an observed mutation and the cidofovir-resistance phenotype be made.

Patients 1 and 3 had an increased IC_{50} of cidofovir while experiencing treatment failure with this drug. In addition, when compared with the IC_{50} for cidofovir in >20 untreated HSV-1 clinical isolates analyzed in our laboratory (2.3 ± 1.5 µg/mL;
Furthermore, the IC\textsubscript{50} decreased by 3.8-fold 2 months after cidofovir therapy was discontinued in patient 1, suggesting that cidofovir resistance was present during therapy. These 2 cases illustrate the difficulty associated with the in vitro diagnosis of cidofovir resistance. Clear cutoff values for the phenotypic diagnosis of cidofovir resistance have not been established; the findings for patients 1 and 3 imply that cidofovir IC\textsubscript{50}s of <10 μg/mL (particularly for HSV-1) may represent significant cidofovir resistance. Interpretation of the results of in vitro resistance assays is further complicated by the intrinsic variability of phenotypic resistance assays. Another peculiarity of cidofovir therapy for HSV infection is that some patients experience treatment failure despite there being apparent phenotypic susceptibility, as illustrated in the first case and in the literature [2].

Two of our patients may have been at increased risk of developing cidofovir-resistant HSV, because they were receiving maintenance doses of cidofovir at the time that HSV disease became apparent. Lower doses of cidofovir may have allowed for low-level viral replication, establishing conditions favorable for the generation of cidofovir-resistant HSV mutants.

In summary, these cases highlight the need for a heightened awareness of antiviral resistance in HSV. Clinicians who care for immunosuppressed patients should now add cidofovir to the list of antiviral medications to which HSV resistance can develop, possibly resulting in an increased risk of disease progression. Studies of the fitness and resistance mechanisms of cidofovir-resistant HSV are required.

Acknowledgments

We thank Julia Clark for technical support.

Financial support. D.L.W. is supported in part by the National Institutes of Health (NIH; training grant ST32DK007038) and the NIH’s Centers for AIDS Research (core grant P30 AI27767).

Potential conflicts of interest. All authors: no conflicts.

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