Development of new cytomegalovirus UL97 and DNA polymerase mutations conferring drug resistance after valganciclovir therapy in allogeneic stem cell recipients

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Abstract

Background: We report on two allogeneic stem cell transplant recipients who developed cytomegalovirus disease associated with new viral mutations that conferred antiviral drug resistance.

Methods: Blood specimens obtained during symptomatic disease were analyzed for mutations in the CMV UL97 and DNA polymerase genes and new mutations were assessed by recombinant phenotyping.

Results: Rising cytomegalovirus (CMV) antigenemia occurred after 4–5 months of preemptive valganciclovir therapy, followed by symptomatic CMV disease including fatal pneumonia in one case. In one case, a new viral UL97 mutation (deletion of codons 601–603) was found which conferred 15-fold increased ganciclovir resistance. In the other case, a known UL97 resistance mutation M460V and a new DNA polymerase (pol) mutation D413A were found. D413A conferred ganciclovir and cidofovir resistance.

Conclusions: Known and newly discovered drug resistance mutations arising during preemptive therapy may complicate post-transplant CMV disease in stem cell recipients. Improved recombinant phenotyping methods enable the rapid quantitation of the resistance conferred by newly identified UL97 and pol mutations.

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1. Introduction

Many stem cell transplant (SCT) centers have adopted a strategy of preemptive antiviral therapy, guided by early detection of CMV reactivation, in order to reduce or prevent morbidity and mortality from subsequent CMV disease (Emery, 2001; Hart and Paya, 2001). Ganciclovir (GCV), and more recently the oral produg valganciclovir (VGCV) are the most commonly used anti-CMV drugs for this purpose. Prolonged drug exposure can lead to GCV resistance, associated with development of characteristic mutations in the UL97 kinase and DNA polymerase (pol) gene regions of the virus. In the SCT setting, the development of GCV resistance is reported to be uncommon and generally limited to case reports (Avery et al., 2004; Erice et al., 1998; Hamprecht et al., 2003; Julin et al., 2002; Prix et al., 1999; Seo et al., 2001), except in the pediatric population where there have been reports of rapid emergence of resistance (Springer et al., 2005; Wolf et al., 1998, 2001). In two adult studies, SCT recipients given 1–3 months of preemptive GCV therapy either did not develop UL97 resistance mutations (Gilbert et al., 2001) or developed low level phenotypic GCV resistance in only one case (Nichols et al., 2001) despite ongoing or rising CMV antigenemia in some individuals. It has also been reported that the use of VGCV for CMV prophylaxis...
in high-risk solid organ recipients resulted in no significant UL97 mutations after use of the drug for at least 100 days (Boivin et al., 2004).

Here, we report the emergence of new resistance mutations in the UL97 and pol genes of CMV in two allogeneic stem cell recipients who had received preemptive treatment with VGCV. Using a recently developed recombinant phenotyping method (Chou et al., 2005), we validated the drug resistance corresponding to these mutations.

2. Materials and methods

2.1. Case reports

Patient 1 was a 52-year-old CMV antibody positive female who received an autologous peripheral blood stem cell transplant for acute myelogenous leukemia (AML). She had a relapse of her AML 2 years later and after re-induction chemotherapy had a mini matched-unrelated-donor stem cell transplant. Negative to low level CMV antigenemia was detected post-transplant and she began taking VGCV 900 mg twice daily and continued for the next 3 months. Low level antigenemia and diarrhea persisted and she developed abdominal pain. Gastrointestinal biopsies were negative for CMV or GVHD. Her prednisone dose was tapered. One month later her CMV antigenemia rose dramatically, and she was switched from VGCV to intravenous foscar-net (FOS). Her antigenemia and GI symptoms persisted and she was switched to intravenous cidofovir (CDV). Repeat duodenal biopsy showed CMV cytopathic effect and CMV antigen. Her CMV antigenemia continued and the CDV was switched to intravenous GCV. She developed dyspnea and chest imaging abnormalities consistent with CMV pneumonitis. A bronchoalveolar lavage was positive for CMV and CMV antigenemia persisted. Both intravenous FOS and CMV hyperimmune globulin therapy were administered, but she expired with refractory respiratory failure.

Patient 2 was a 51-year-old CMV antibody positive female with a history of multiple myeloma and plasma cell leukemia who received a mini matched-unrelated-donor stem cell transplant. When CMV antigenemia developed, VGCV was given preemptively at a dose of 900 mg twice daily. She had an initial response with a decline of antigenemia to undetectable but after 3 months of treatment a rising antigenemia was observed. She received daily intravenous FOS infusions for a total of 30 days and an antigenemia assay performed approximately 2.5 months after discontinuing foscar-net was negative.

No ganciclovir pharmacokinetic monitoring was done for either patient.

2.2. Genotypic susceptibility testing

The CMV genotype was determined from viral sequences directly amplified from blood specimens and cell pellets. CMV DNA for sequencing analysis was extracted from patients’ specimens using the Roche MagNA Pure and the corresponding total nucleic acid kit. Purified nucleic acids were then amplified in three separate reactions; one reaction created an amplicon spanning codons 450–625 of the UL97 gene, while the second and third reactions created amplicons that combined to span codons 275–1000 of the UL54 gene. Cycle sequencing was carried out on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer’s recommendations, using BigDye Version 3.1 sequencing kits. Raw sequence data was base-called and trimmed using the instrument’s Sequencing Analysis 3.7 software, and the sequence was then assembled into a contig and edited using Sequencher software (Gene Codes Corporation, Ann Arbor, MI).

2.3. Transfer of viral mutations to a reference CMV strain

Laboratory CMV strain AD169 was used to derive a recombinant strain T2211 containing a secreted alkaline phosphatase (SEAP) reporter gene and unique restriction sites in UL97 and pol to facilitate the construction of mutant viruses, as previously described (Chou et al., 2005). UL97 or pol mutations were introduced by restriction digestion of T2211 DNA and co-transfection with an overlapping DNA segment containing the mutation of interest (Chou et al., 2005). Live virus that resulted from the co-transfection was screened by PCR, restriction digestion and sequencing. Once a recombinant screened positive for the intended mutations, it was plaque purified and the genotype verified by sequencing the entire coding sequence of the mutated gene (UL97 or pol) (Chou et al., 2002, 2003).

2.4. Susceptibility testing of recombinant mutant viruses

Yield reduction assays for drug resistance were performed as recently published (Chou et al., 2005). High titer extracellular virus stocks were prepared from plaque-purified recombinant virus. Twenty-four-well plates of confluent human foreskin fibroblasts were inoculated with the virus to be tested at a multiplicity of infection (MOI) of 0.015. After a 90-min incubation period, the inoculum was removed and replaced with media containing 2-fold serial dilutions of one of the three antiviral drugs (GCV, FOS or CDV). Five to six days after inoculation, supernatant from the culture wells were assayed for SEAP activity by chemiluminescence (relative light units, RLU) (Chou et al., 2005). The concentration of drug required to reduce supernatant SEAP activity (RLU) by 50% (IC50) was determined by curve fitting and compared with control parental viruses and viruses containing known resistance mutations. Control viruses included parental strains T2211 and T2233, along with T2259, a previously constructed strain containing the established UL97 M460V mutation (Chou et al., 2005). A minimum of five replicates meeting established quality control criteria were
Fig. 1. Time course of clinical and virological developments. BAL, bronchoalveolar lavage; IVIG, intravenous immunoglobulin; CMV antigenemia expressed as number of pp65-antigen positive cells per 200,000 leukocytes.
obtained, and results reported as mean and standard error of the mean values.

2.5. Comparison of growth properties of recombinant viruses

Viral (SEAP) growth properties were assessed by inoculating 24-well plates of confluent human foreskin fibroblasts with the virus stocks to be compared, at MOI of 0.015. Aliquots of supernatant were collected on days 1, 4–8 and were assayed for SEAP activity as previously described (Chou et al., 2005). The experiments were set up in quadruplicate and simultaneously to reduce variation caused by differences in cell cultures. The SEAP activity at each time point was graphed as the mean RLU and standard error of the mean.

3. Results

3.1. Clinical features of drug resistance

Both patients had an initial response to VGCV with clearing of CMV antigenemia but it recurred after 3–4 months on therapy (Fig. 1), associated with gastrointestinal symptoms in both cases. Rising antigenemia prompted the selection of FOS as alternative therapy, which was successful in one instance (case 2). In the other case, a sharply rising antigenemia culminated in the development of a fatal CMV pneumonitis despite the administration of multiple antiviral drugs in succession.

3.2. Genotypic analysis of clinical specimens

Pretreatment specimens were not available from either patient. Genotypic antiviral resistance testing was done on clinical specimens from both patients when recurrent antigenemia led to a suspicion of drug resistance. From case 1, CMV sequences amplified from a blood specimen demonstrated a UL97 mutation (deletion of codons 601–603; del601-3) but no pol mutations. The UL97 mutation was seen after approximately 5 months of oral VGCV. This mutation has not previously been confirmed as a GCV-resistance mutation but is located in a region of UL97 (codons 590–607) that is the locus of other GCV-resistance mutations (Chou et al., 2002).

Sequencing of a CMV isolate from both blood and cell pellet of patient 2 obtained prior to the start of FOS therapy revealed a previously described GCV-resistance mutation (Chou et al., 1995) in UL97 (M460V) and a newly recognized mutation in pol (D413A). Both mutations were detected in both the blood and cell pellet sequence as a mix with wild-type virus sequence, though the D413A configuration was dominant. The patient had received approximately 3.5 months of oral VGCV prior to detection of the mutation.

3.3. Antiviral susceptibility of recombinant viruses

Previously uncharacterized mutations UL97 del601-3 and pol D413A were introduced into reference strain T2211, resulting in recombinant strain T2325 containing the UL97 mutation del601-3, and strain T2294 containing the pol mutation D413A.

SEAP yield reduction assays were performed on the recombinant strains and controls, with results as shown in Table 1. The UL97 del601-3 mutation conferred 15-fold GCV

<table>
<thead>
<tr>
<th>CMV strain</th>
<th>Genotype</th>
<th>IC50 by SEAP assay a</th>
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<tbody>
<tr>
<td></td>
<td>UL97</td>
<td>pol</td>
</tr>
<tr>
<td>T2211</td>
<td>wt (SwaI)</td>
<td>wt (PmeI)</td>
</tr>
<tr>
<td>T2233</td>
<td>wt</td>
<td>wt (PmeI)</td>
</tr>
<tr>
<td>T2259</td>
<td>M460V</td>
<td>wt (PmeI)</td>
</tr>
<tr>
<td>T2294</td>
<td>wt (SwaI)</td>
<td>D413A (PmeI)</td>
</tr>
<tr>
<td>T2325</td>
<td>del601-3</td>
<td>wt (PmeI)</td>
</tr>
</tbody>
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Resistant values shown in bold. wt, wild type; SwaI, HS87Y change associated with SwaI restriction site; PmeI, S897L change associated with PmeI restriction site.

a Mean (μM) ± standard error of the mean.
resistance over baseline, without FOS or CDV resistance. The D413A mutation conferred GCV-CDV double resistance without resistance to FOS.

3.4. Growth properties

Multicycle growth curves (Fig. 2) showed that strain T2294 containing pol mutation D413A was modestly but significantly growth impaired, showing a 0.7–1 log reduction in SEAP activity at 5–6 days post-inoculation.

4. Discussion

Despite the impression that therapy for CMV in SCT recipients infrequently leads to resistance, we observed the development of known and new viral resistance mutations in two cases, in association with rising antigenemia and ensuing morbidity and mortality. The frequency of CMV drug resistance in the transplant population appears to vary considerably according to the tissue type and recipient risk factors (Cope et al., 1997a,b). The substantial GI symptoms, including diarrhea in both patients, may have decreased absorption of VGCV and enhanced the development of resistance.

GCV resistance in CMV is usually caused by mutations in the UL97 kinase gene, which impair the initial phosphorylation of the drug that is necessary for its antiviral activity. The known mutations are tightly clustered at codons 460, 520 and 590–607 (Chou et al., 2002). M460V is a frequent and well-known GCV-resistance mutation, as found in case 2. The mutation by itself confers ∼8-fold increased GCV resistance (IC50 value) over a baseline sensitive virus (Chou et al., 2002). There have been several other observations of deletion mutations (e.g. 590–600, 597–603) that have not been validated by recombinant phenotyping (Wolf et al., 2001). Given that the entire codon range 591–607 can be deleted from UL97 without much effect on viral growth (Chou et al., 2002), there are likely to be many ways in which this region can be mutated in order to develop GCV resistance of varying degrees. Most likely, this part of UL97 is involved in recognition of GCV as a substrate.

Mutations in the viral DNA polymerase can confer resistance to any or all of the current systemic CMV antivirals and can be selected after exposure to any of them (Chou et al., 2003). The pol D413A mutation observed in case 2 was shown to confer dual GCV-CDV resistance. It is located in pol exonuclease domain II, where a previously described mutation D413E was found to confer GCV and CDV resistance and FOS hypersensitivity (Chou et al., 2003). Unlike D413E, D413A is a nonconservative amino acid substitution and was an unexpected finding. Codons 408 and 413 are highly conserved residues involved in magnesium binding and functional exonuclease activity (Chou et al., 2003). D413A was shown to grow more slowly than the control strain T2233 indicating that pol mutations may lead to growth impairment (Fig. 2). Despite possible failure of foscarnet therapy in case 1, no FOS resistant mutation was detected. However, pol sequencing was restricted to codons 275 through 1000 and it is possible that a resistance mutation was present outside this region in UL54. There is no residual specimen from patient 1 to allow further sequencing. To date, however, no FOS resistance mutations have been mapped beyond codon 1000 and most genotypic testing done for clinical purposes does not extend beyond pol codon 1000.

Specimens from case 2 contained mutations in both the UL97 (M460V) and pol (D413A) genes. If both mutations reside on the same viral genome, the combination of UL97 and pol mutations is expected to confer higher level GCV resistance than either mutation independently (Smith et al., 1997), as was recently illustrated by recombinant phenotyping with another combination of UL97 and pol mutations (Chou et al., 2005). In the present case, because each mutation was detected as a mixture with its wild type counterpart, it is difficult to determine if both mutations were on the same viral genome.

In transplant medicine, the use of preemptive treatment for CMV infection has become more convenient because of the oral bioavailability and antiviral efficacy of VGCV. However, drug toxicity, delayed onset of disease after discontinuing therapy, and antiviral drug resistance are limiting factors. The cases here illustrate the need to monitor for resistance when rising viral loads or viral shedding occur after several weeks to months on therapy. Because rising viral loads or persistent shedding are not necessarily due to drug resistance, genotypic testing may provide timely confirmation and guidance as to applicable alternate therapy. Since many rapid diagnostic methods in use today do not yield a live viral isolate, recombinant phenotyping as done here will become the usual way to quantitate the resistance of newly recognized viral mutations.

Conflicts of interest

None.

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References


