GANCICLOVIR RESISTANCE MUTATIONS IN UL97 AND UL54 GENES OF HUMAN CYTOMEGALOVIRUS ISOLATES RESISTANT TO GANCICLOVIR

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Summary. – Human cytomegalovirus (HCMV) resistance to ganciclovir results from mutations in viral phosphotransferase (UL97) and/or DNA polymerase (UL54) genes. The HCMV isolates from the blood of immunocompromised patients with persisting presence of the pp65 antigen in the blood in spite of ganciclovir therapy were tested for ganciclovir susceptibility by an immediate-early antigen plaque reduction assay, and the UL54 and UL97 genes were sequenced. Nine isolates from eight patients (six patients with acquired immune deficiency syndrome (AIDS), one liver transplant recipient and one renal transplant recipient) showed phenotypic resistance to ganciclovir. All these ganciclovir-resistant HCMV isolates harbored one or more of the following UL97 mutations: M460V, A594V, A594T, L595S, C603W, and M615V. Two isolates harbored the P522S mutation in the UL54 gene. The M615V mutation in the UL97 gene has not been reported earlier and its role in ganciclovir resistance remains to be elucidated. In ganciclovir-resistant HCMV isolates the UL54 gene was less frequently mutated than the UL97 gene. The P522S mutation was relatively frequent in UL54-mutated HCMV isolates.

Key words: Human cytomegalovirus; resistance; ganciclovir; UL54; UL97

Introduction

Ganciclovir, an antiviral agent active against HCMV, is widely used for the treatment of HCMV infections. In HCMV-infected cells, the phosphotransferase encoded by the HCMV UL97 gene is responsible for initial phosphorylation of ganciclovir to its monophosphate which is converted to ganciclovir triphosphate by cellular kinases. Ganciclovir triphosphate acts as an inhibitor and a substrate for the DNA polymerase encoded by the HCMV UL54 gene; this results in the inhibition of HCMV DNA synthesis and termination of DNA elongation (Matthews and Boehme, 1988). In severely immunocompromised patients, such as recipients of solid organ or bone marrow transplants, or in patients with AIDS, the treatment of HCMV-associated diseases often necessitates a prolonged administration of ganciclovir. However, it has been shown that (i) a prolonged ganciclovir treatment can promote the development of ganciclovir-resistant HCMV strains resulting in an impaired response to the therapy (Erice *et al*., 1989; Drew *et al*., 1991), and (ii) the resistance to ganciclovir results from the development of mutations in the UL97 and UL54 genes (Erice, 1999).

The aim of the present study was to identify the UL97 and UL54 gene mutations in ganciclovir-resistant HCMV isolates obtained from immunocompromised patients.

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Abbreviations: AIDS = acquired immune deficiency syndrome; EMEM = Earle's Minimum Essential Medium; FCS = fetal calf serum; HCMV = Human cytomegalovirus; IC_{50} = 50% inhibitory concentration

Materials and Methods

Patients, blood samples, assay of HCMV-pp65 antigen, and virus isolation. Assay of the HCMV-pp65 antigen in the blood was performed as described by Reynes *et al*. (1996). An additional heparinized blood sample for HCMV isolation was collected from patients positive for HCMV-pp65 antigen in the blood. HCMV was grown in MRC-5 cell monolayers maintained in Earle's Minimum Essential Medium (EMEM) supplemented with 10% of fetal calf serum (FCS), 2 mmol/l glutamine, 150 U/ml penicillin, and 8 µg/ml gentamicin in 25 cm˛ tissue culture flasks. The latter were seeded with approximatively 5 x 10^6 buffy coat cells. The cultures maintained at 37°C in EMEM supplemented with 2.5% FCS were examined for viral cytopathic effect (CPE) twice weekly for three weeks.

Phenotypical resistance assay. Susceptibility of HCMV to ganciclovir was determined by an immediate-early antigen plaque reduction assay described by Abraham *et al*. (1999). The HCMV isolates with the 50% inhibitory concentration (IC_{50}) of ganciclovir below 6 µmol/l were considered susceptible to ganciclovir, those with the IC_{50} >12 µmol/l were considered resistant; and those with IC_{50} >6–12 µmol/l were considered intermediate (Landry *et al*., 2000).

UL 97 and UL54 gene sequencing. DNA was extracted from cell cultures using the QIAamp DNA Mini Kit (Qiagen, France). DNA fragments from the UL97 and UL54 genes were amplified by PCR. The reaction mixture (50 µl) contained 0.5 µmol/l primers, 200 µmol/l dNTPs, and 0.5 U of Taq DNA polymerase. The PCR consisted of an initial denaturation step at 95°C for 5 mins, 35 cycles of 95°C/30 secs (denaturation), 52°C/30 secs (annealing), and 72°C/1 min (extension), and a final extension step at 72°C for 10 mins. PCR products were purified using the Qiagen PCR Purification Kit and sequenced using the ABI Prism BigDye Terminator v 1.1 Cycle Sequencing Kit and an ABI 310 Automated DNA Sequencer (Applied Biosystems, France). The UL97 gene (codons 400–707) was amplified using the primers HLF97-F and HLF97-R (Lurain *et al*., 2001). The forward strand was sequenced using the primers 5'-ATCGACAGCTACCGACGTGCC-3' (nucleotides 1285–1305 on AD169 strain) and 3'-GTCGGAGCTG TCGGCGCTGGG-5' (nucleotides 1650–1670), while the reverse strand was sequenced using the primers 5'-CGACACGAGGA CATCTTG-3' (nucleotides 1934–1917) and 3'-CCCAGCGCCGA CAGCTCCGAC-5' (nucleotides 1670–1650). Two fragments of the UL54 gene were amplified and sequenced. The first fragment (codons 350–579) was amplified using the primers UL54.1 F1 and UL54.1 R1 and was sequenced using the primers UL54.1 F1, UL54.1 F2, UL54.1 R1, and UL54.1 R2 (Table 1). The second fragment (codons 680–1005) was amplified using the primers UL54.2 F3 and UL54.2 R3 and was sequenced using the primers UL54.2 F3, UL54.2 F4, UL54.2 R4, and UL54.2 R3. These fragments encompassed the UL97 and UL54 gene catalytic domains within which the ganciclovir-resistance mutations have been localized (Erice, 1999). Coding and non-coding DNA sequences were aligned, the consensus sequence was computed and translated into an amino acid sequence. The amino acid sequence of each fragment was aligned and compared with the corresponding sequences of the HCMV strain AD169; sequence alignments and comparisons were done using the Sequence Navigator software. The UL54 gene mutations known to result from natural polymorphism, such as A885T, A885S, T insertion at codon 885, P887S, S897L, and N898D (Chou *et al*., 1999), were not taken into consideration.

Results

Between January 1997 and October 2002, 214 immunocompromised patients with HCMV disease treated with ganciclovir were followed up; they were 74 patients with AIDS, 91 renal transplant recipients and 49 liver transplant recipients.

Presence of the HCMV-pp65 antigen was assayed in the blood of these patients between13 and 23 days after initiation of the therapy. These assays revealed 17 positive patients with the positivity ranging from 1 to 1400 positives/2 x 10^5 leukocytes.

The virus isolation experiments were positive with 12 patients, who yielded 14 virus isolates. The latter were tested for ganciclovir resistance. Nine of these patients had AIDS, their CD4 cell counts ranged from 1 to 48 cells/mm^3 (median, 12 cells/mm^3) and they presented a HCMV retinitis. The other three patients were organ transplant recipients (two liver transplant recipients and one renal transplant recipient), who presented a primary HCMV infection. The 3 organ transplant recipients had received a long-term oral ganciclovir prophylaxis (Kletzmayr *et al*., 2000).

Table 1 Primers used for UL54 gene amplification and sequencing

Primer	Sequence (5'→3')	Start/stop codon positions[1]
UL54.1 F1 (sense)	CACTTCGGAGGGTGTGATCT	1050–1069
UL54.1 R1 (antisense)	TCTGCTGTCCGTCAAAGATG	1738–1719
UL54.1 F2 (sense)	TTTCTTTTTACACAGCCCCG	1326–1345
UL54.1 R2 (antisense)	CGGGGCTGTGTAAAAAGAAA	1345–1326
UL54.2 F3 (sense)	GCGTTTCCAACGACAATCAC	2039–2058
UL54.2 R3 (antisense)	ATCCTCAAAGAGCAGCGAGA	3015–2996
UL54.2 F4 (sense)	AACGGTATGATGCCGTG CT	2473–2492
UL54.2 R4 (antisense)	AGACACGGCATCATACCGTT	2492–2473

[1]Acc. No. X17403.

Table 2. UL54 and UL97 gene mutations in HCMV isolates from ganciclovir-treated patients

Patient/ isolate No.	Clinical category	Ganciclovir treatment (days)[a]	HCMV-pp65 antigen[b]	IC_{50} (μmol/l)	UL54	UL97
1	AIDS	155	38	71.0	WT	M460V
2	AIDS	13	48	4.9	WT	WT
3	AIDS	83	59	9.2	WT	WT
4 (1)	AIDS	224	118	19.2	WT	A594T
(2)		306	1400	22.5	P522S	A594T
5	AIDS	381	29	89.0	WT	A594V, M615V
6 (1)	AIDS	21	910	5.5	WT	WT
(2)		164	120	63.0	WT	A594V
7	AIDS	219	282	53.0	P522S	L595S
8	Liver tr.	112	262	22.5	WT	A594V
9	AIDS	135	374	80.0	WT	M460V
10	AIDS	283	2	7.1	WT	WT
11	Liver tr.	115	67	4.7	WT	WT
12	Renal tr.	120	2500	13.4	WT	C603W

[a]Cumulative duration of induction and maintenance treatments.
[b] The presence of HCMV-pp65 antigen in the blood. No. of positives of 2 x 10^5 leukocytes.
Liver tr. = liver transplantation.
Renal tr. = renal transplantation.

The ganciclovir resistance experiments showed (Table 2) that 9 CMV isolates from 8 patients (6 patients with AIDS, liver transplant recipient and 1 renal transplant recipient) were found phenotypically resistant to ganciclovir. All the ganciclovir-resistant isolates harbored mutations in the UL97 gene. A mutation in codon 594 was identified in 4 patients: 3 patients had the A594V mutation and 1 patient (2 isolates) had the A594T mutation. The M460V mutation was identified in 2 patients and the mutations L595S, C603W and M615V were each identified in 1 patient, the M615V mutation being associated with the A594V mutation (patient No. 5).

Only 2 patients harbored a mutation in the UL54 gene, namely the P522S mutation.

This mutation was combined with the A594T mutation in 1 patient (patient No. 4) and with the L595S mutation in another (patient No. 7). In patient No. 4, the development of the UL97 gene mutation preceded the development of the UL54 gene mutation. The IC_{50} values obtained from the 2 isolates with mutations in both the UL54 and UL97 genes seem to be not different from those obtained from the 7 isolates with mutations only in the UL97 gene. A definite conclusion concerning this issue will require statistical analysis of a larger number of isolates.

Discussion

The responsibility of the UL54 and/or UL97 gene mutations for the HCMV resistance to antiviral drugs in general and the involvement of numerous mutations in the HCMV resistance to ganciclovir in particular is well established (Erice, 1999). Unfortunately, numerous studies published on this subject are often based on a relatively small number of resistant HCMV isolates. Therefore, it remains important to gain additional more complete data on genotypical HCMV resistance.

The UL97 gene mutations M460V, A594V, A594T, L595S, and C603W identified in the present study have been already described (Baldanti *et al*., 1996; Chou *et al*., 1995, 1997; Erice *et al*., 1997). In contrast to the A594T and C603W mutations, the M460V, A594V, and L595S mutations have been the most frequently reported ones (Erice, 1999). The role of these UL97 gene mutations in conferring resistance to ganciclovir has been confirmed by marker transfer experiments (Gilbert *et al*., 2002). The fact that UL97 gene mutations in other codons, such as codons 520, 591, 592, 596, 607, and 659, as well as UL97 gene codon deletions have a role in the ganciclovir resistance, is also well established (Gilbert *et al.*, 2002). However, the sample size of the ganciclovir-resistant HCMV isolates analyzed in the present study is too small to be representative of the UL97 gene mutation diversity.

The M615V mutation has not been reported earlier. We observed this mutation in combination with the A594V mutation in an HCMV isolate highly resistant to ganciclovir (IC_{50} = 89.0 μmol/l). Therefore, although codon 615 is located outside the catalytic domain of UL97, the M615V mutation could be involved in ganciclovir resistance. However, the possible role of this mutation in ganciclovir resistance remains to be established. Whereas all the ganciclovir-resistant HCMV isolates harbored mutations in the UL97 gene, the UL54 gene was less frequently mutated. This observation, in accordance with the literature, highlights the major contribution of UL97 gene mutations to the ganciclovir resistance. Indeed, in the

absence of phosphorylation by the UL97 gene product, ganciclovir remains inactive.

The P522S mutation in the UL54 gene has been identified earlier in clinical HCMV isolates (Jabs *et al*., 2001), as well as in ganciclovir-resistant recombinant mutant viruses generated from overlapping DNA fragments (Cihlar *et al*., 1998). This mutation that represented the sole UL54 gene mutation identified in the present study was found in 25% (2/8) of the patients infected with ganciclovir-resistant HCMV strains. This observation indicates that P522S could represent a frequent UL54 gene mutation developed in response to ganciclovir therapy.

The A594T mutation in the UL97 gene was identified in the first HCMV isolate obtained from patient No. 4, while the P522S mutation in the UL54 gene was identified in a subsequent HCMV isolate; the development of this gene mutation was not associated with a notable increase in HCMV phenotypic resistance. Furthermore, the IC_{50} values obtained with the isolates mutated in both the UL54 and UL97 genes were not different from those obtained with the isolates mutated only in the UL97 gene. These observations suggest that the P522S mutation has a modest impact on HCMV phenotypic resistance to ganciclovir.

Our study also confirms the possible role of oral ganciclovir prophylaxis administered to organ transplant recipients in the development of ganciclovir resistance (Isada *et al*., 2002; Limaye, 2002). Indeed, two patients who had received this post-transplantation treatment developed a primary HCMV infection with isolation of a ganciclovir-resistant HCMV isolate harboring UL97 mutations.

The patients infected with ganciclovir-resistant HCMV isolates harboring UL97 gene mutations can benefit from treatment with foscarnet, since this drug inhibits the HCMV DNA polymerase in the absence of preliminary phosphorylation. The resistance to foscarnet is associated with UL54 gene mutations. Nevertheless, the P522S mutation identified in the present study does not seem to confer the foscarnet resistance (Cilhar *et al.*, 1998).

In conclusion, in accordance with previous studies, our results confirm predominant role of the UL97 gene in the HCMV resistance to ganciclovir and indicate that the P522S mutation could represent one of the most frequent UL54 gene mutations in ganciclovir-resistant HCMV isolates.

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