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GCV resistance due to the mutation A594P in the cytomegalovirus protein UL97 is partially reconstituted by a second mutation at D605E

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Abstract

A ganciclovir (GCV)-resistant human cytomegalovirus (HCMV) was isolated from an AIDS patient. Molecular analysis of the HCMV UL97 gene revealed two point mutations, A594P and D605E, respectively. In order to evaluate quantitatively the impact of the individual mutations on GCV phosphorylation, recombinant vaccinia viruses (rVVs) were generated carrying either the two mutations (rVV-594/605) or only one mutation (rVV-594 or rVV-605, respectively). In cells infected with the rVV-594/605 double mutant, the GCV phosphorylation was decreased to 50% compared with the phosphorylation in cells infected with the rVV-UL97 wild-type. In cells infected with the rVV-594, however, the GCV phosphorylation was further decreased to 30%. Interestingly, the mutation D605E led to an even better GCV phosphorylation than that measured in cells infected with the rVV-UL97 wild type. These results were confirmed by plaque reduction assays, indicating that rVV-594 was more resistant to GCV than rVV-594/605. In contrast, rVV-605 was more sensitive to GCV than the rVV-UL97 wild type. Therefore, our results demonstrated for the first time that compensatory mutations can also occur in HCMV, as already shown for human immunodeficiency virus type 1.

Keywords: Cytomegalovirus; Ganciclovir; Phosphorylation UL97; Recombinant vaccinia viruses; Compensatory mutations

1. Introduction

Human cytomegalovirus (HCMV) infections are a significant cause of morbidity and mortality in immunocompromised hosts such as patients with acquired immune deficiency syndrome (AIDS) or transplant recipients (Drew et al., 1991;

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Knox et al., 1991; Erice et al., 1989). Ganciclovir (GCV) is the drug of choice for the treatment of HCMV disease and also for prophylaxis in groups at high risk for HCMV diseases. Long-term treatment with anti-HCMV drugs can lead to the production of drug-resistant mutants (Erice et al., 1989; Stanat et al., 1991). GCV, a nucleoside analogue, requires monophosphorylation by the virus-encoded UL97 phosphotransferase (Sullivan et al., 1992); subsequent phosphorylations are carried out by cellular enzymes. GCV triphosphate is the active form that is a competitive substrate/inhibitor of the viral DNA polymerase. During GCV treatment, GCV-resistant mutants can result from mutation(s) either in the UL97 phosphotransferase gene or the DNA polymerase gene (UL54), or both (Baldanti et al., 1998; Chou et al., 1995b; Mendez et al., 1999; Wolf et al., 1998). However, recently it has been shown that GCV-resistant HCMV strains can also be selected by aciclovir (Michel et al., 2001). By using marker transfer experiments, some mutations in the UL97 gene have been confirmed to be responsible for GCV resistance (Baldanti et al., 1995; Chou et al., 1995a; Khan et al., 1998; Lurain et al., 1994; Wolf et al., 1995). However, this procedure of recombinant HCMV generation is laborious and time-consuming. Furthermore, the method is dependent on a certain loss of GCV-phosphorylating activity for selection of recombinants. Metzger et al. (1994) have established a recombinant vaccinia virus (rVV) system in which the UL97 coding region of HCMV was introduced into naturally GCV-resistant vaccinia virus. The vaccinia virus system has been used for the characterization of the pUL97-dependent GCV phosphorylation (Michel et al., 1996, 1998, 1999; Wagner et al., 2000), as well as to test the phosphorylation of the antiviral drugs aciclovir and penciclovir by pUL97 (Zimmermann et al., 1997). More recently, the rVV system has been used to analyze the quantitative effect of ten different UL97 mutations identified in the UL97 protein of GCV-resistant clinical HCMV isolates (Baldanti et al., 2002). Using this rVV system, we analyzed a clinical HCMV isolate that had compensatory mutations in the UL97 phosphotransferese gene.

2. Materials and methods

2.1. Cells and viruses

Human embryonic lung fibroblasts (HEL) were grown in Eagle's minimum essential medium (MEM: Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% fetal bovine serum (FBS, Flow Laboratories Inc., Irvine, VA), kanamycin (60 μg/ml), and 0.12% $NaHCO_3$. Maintenance medium contained 5% instead of 10% FBS. HEL cells were used for isolation, propagation and titration of HCMVs. The HCMV isolate, S.I strain, was recovered from the urine of a patient with AIDS who underwent multiple sequential courses of GCV treatment for HCMV colitis. Since GCV-sensitive corresponding to wild type virus could not be isolated from the patient, the laboratory strain, AD169, was used as a reference strain in some experiments. CV-1 cells and 143B thymidine kinase (TK)-deficient cells were also grown as mentioned above and were used for transfection and infection experiments of vaccinia virus, as described previously (Metzger et al., 1994; Michel et al., 1996, 1999).

2.2. Susceptibility test of HCMV and rVVs to GCV

GCV was a gift from Wellcome Japan, Kobe. The susceptibility of the S.I strain to GCV was determined by a plaque reduction test using HEL cells, as described previously (Harada et al., 1997; Khan et al., 1998). Laboratory strain AD169 was included as a GCV-sensitive control. For GCV susceptibility assay of rVVs, monolayers of 143B TK-deficient cells in 35 mm plastic dishes (Iwaki Glass Co., Funahashi, Japan) were inoculated with approximately 50 plaque-forming units (PFU) of individual rVVs. After adsorption at room temperature for 60 min, the dishes were overlaid with maintenance medium containing different concentrations (50–1000 μM) of GCV. Duplicate dishes were used for each concentration of GCV. The inhibitory concentration (IC_{50}) was defined as the concentration of GCV that produced 50% reduction in plaque numbers compared with those in the control dishes.

2.3. Single strand conformation polymorphism (SSCP) analysis and sequencing

HCMV DNA was prepared according to the Hirt's procedure (Hirt, 1967). The two overlapping fragments of the UL97 coding region were amplified by two primer sets. Each primer was labeled with biotin at 5′-end for non-radioisotopic SSCP analysis and sequencing. The sequences of the primer pairs were as follows: 5′-TACGGCGTTATTGCATGT-3′ and 5′-ATTCGTGCAGCATGGTCT-3′ for fragment I (nucleotide number 1634–1990) and 5′-ATCACCAGTGTCGTGATAGC-3′ and 5′-CGACATGCAATATCGAAGTA-3′ for fragment II (nucleotide number 1340–1654). For SSCP analysis and sequencing, the PCR product was purified from agarose gel using QIAXII Extraction Kit (QIAGEN GmbH and QIAGEN Inc, Hilden, Germany). SSCP analysis and sequencing were performed using ΔTth DNA polymerase Sequencing High-Cycle kit and Imaging High-Chemiluminescence Detection kit (Toyobo Co, Osaka), as described previously (Harada et al., 1997; Khan et al., 1998).

2.4. Generation of rVV containing mutations in UL97 gene

The p7.5K-UL97 plasmid was constructed by insertion of UL97 coding sequence of AD169 into the multiple cloning site of vaccinia virus expression vector p7.5K131, as described previously by Metzger et al. (1994). To clone the mutated region of the S.I strain, a 618 bp 3′-terminal fragment of UL97 sequence from the S.I strain was amplified by PCR using primer pair, pri.5-KpnI (5′-CAG GAG ACG GGT ACC GCG CGC CG-3′) and pri.6-EcoRI (5′-AAC TGT TCC CCG ACT AAA TCG ATA GAA TTC AAG-3′). After digestion with *Kpn*I and *Eco*RI, the amplified fragment was inserted into p7.5K-UL97 plasmid in which KpnI–EcoRI fragment had been deleted by digestion with both restriction enzymes. The vaccinia expression vector was used to produce vaccinia recombinants according to standard protocols (Mackett et al., 1982). The rVV containing two mutations was designated rVV-594/605. As a control, the rVV-UL97 wild-type (WT) that contained the UL97 gene of AD169 was used throughout the experiments (Metzger et al., 1994; Michel et al., 1996, 1999).

2.5. In vitro site-directed mutagenesis

The 618 bp KpnI–EcoRI fragment from the S.I strain was cloned into pUC18 plasmid. Each mutation in this KpnI–EcoRI fragment was reverted to wild-type sequence by ExSite PCR-based site-directed mutagenesis kit (Stratagene Ltd, Cambridge, UK) according to the method described by Weiner et al. (1994). Briefly, plasmid DNA was added to a 25 μl PCR mixture containing the following reagents: mutagenesis buffer; one pair of mutagenic primers (Fig. 1), (one of them was phosphorylated at the 5′-end); dNTP mixture; and Exsite DNA polymerase blend. The cycling conditions were as follows: one cycle of 4 min at 94 °C, 2 min at 56 °C, and 1 min at 72 °C; followed by eight cycles of 1 min at 94 °C, 2 min at 56 °C and 1 min at 72 °C; and a final cycle of 5 min at 72 °C. Following completion of the PCR, the template plasmid DNA was digested with *Dnp*I restriction enzyme. The undigested linear DNA was then treated with cloned Pfu DNA polymerase (Stratagene Ltd, Cambridge, UK) to remove extended bases on the 3′-end of the PCR product. The linear DNA was intramolecularly ligated with T4 ligase at 37 °C for 1 h. The ligated DNA was then digested with *Eco*RI and *Kpn*I. The KpnI–EcoRI fragment was inserted to p7.5K-UL97 plasmid in which wild-type KpnI–EcoRI fragment had been deleted. The individual rVVs were constructed as mentioned above. The sequence of individual rVVs was confirmed by using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit and ABI Prism 310 Genetic Analyzer. The rVVs with single mutation at codons 594 and 605 were designated as rVV-594 and rVV-605, respectively.

2.6. Analysis of UL97 protein

Western blot analysis for detection of pUL97 expressed by recombinant vaccinia viruses was performed as previously described using a pUL97-

specific polyclonal antiserum (Michel et al., 1996, 1998, 1999). CV-1 cells were mock-infected or infected with the different vaccinia viruses at an MOI (multiplicity of infection) of five and harvested 24 h postinfection. Total-cell extracts were resolved by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to the nitrocellulose membranes. The membranes were reacted with anti-UL97 rabbit serum (dilution of 1:300) followed by incubation with secondary donkey anti-rabbit IgG conjugated with horseradish peroxidase (dilution of 1:1000). The bound antibody was detected using the ECL Western blot detection kit (Amersham International Inc, Amersham, UK) according to the instructions of the Manufacturer.

2.7. Analysis of GCV phosphorylation

The TK-deficient 143B cells were infected with individual UL97-expressing rVVs at an MOI of 10. At 1 h post infection, 5 μCi of [^{3}H]-GCV was added at a specific activity of 100 mCi/mmol. After 24 h of incubation, cells were harvested and washed three times with PBS. The cells were suspended in PBS and treated with 0.5 M perchloric acid. The extracts were adjusted to pH 6.5 with 2.5 M KOH in 1.5 M KH_2PO_4 and centrifuged for 5 min at $14\,000 \times g$, and the supernatants were used for high-pressure liquid chromatography (HPLC). HPLC was performed on a Prep Pac reversed-phase column (250 by 4.6 mm; Pharmacia, Uppsala, Sweden) with 20 mM KH_2PO_4 (pH 6.0) 7.5% methanol as the mobile phase, using isocratic elution at a flow rate of 1 ml/min. Fractions containing the GCV-phosphates (mono-, di- and triphosphates) and unphosphorylated compound were collected according to their retention times as determined by external standard runs. The radioactivity of each fraction was determined by liquid scintillation counting. The amount of phosphorylated compound was normalized for 1×10^5 cells.

3. Results

3.1. Susceptibility of S.I strain to GCV

The susceptibility of laboratory strain AD169 and the clinical HCMV isolate S.I to GCV were determined by the plaque reduction test. The IC_{50} for the S.I strain was 6.0 μM that is six times higher than that measured for AD169.

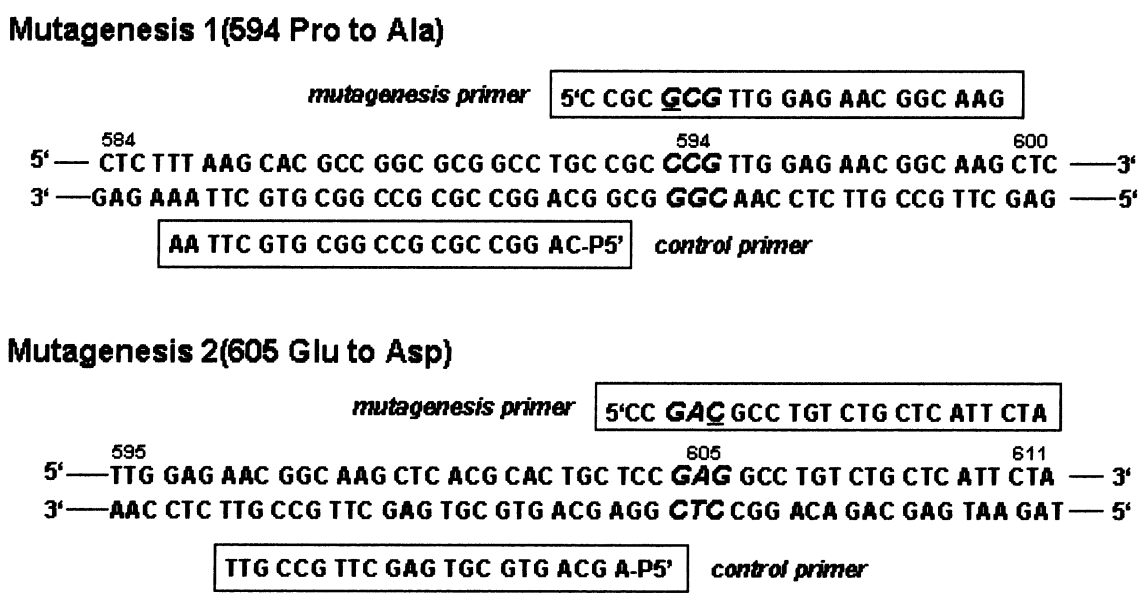

Fig. 1. Primer sequences for PCR based site directed mutagenesis.

3.2. Molecular analysis of UL97 coding region of the S.I strain

In order to identify mutations in the UL97 region, SSCP analysis was carried out using the PCR products amplified from the S.I isolate as well as from the laboratory strain AD169. Differences in the mobility were found only in a fragment spanning from nucleotide number 1634 to 1990. Sequencing of the region revealed two point mutations, one at position 1780 (G to C) and a second at 1815 (C to G), both resulting in amino acids substitutions. Although substitutions at codon 594 are often observed in clinical isolates, A594P and D605E have not been previously reported. To exclude the possibility of any mutation in the UL54 gene, seven overlapping fragments were also examined by PCR-SSCP analysis as described previously (Harada et al., 1997). No differences in mobility between AD169 and S.I strain were observed (data not shown).

3.3. Phosphorylation of GCV in the cells infected with rVVs

In order to further elucidate the influence of both mutations on the GCV-resistance, recombinant vaccinia viruses were generated. Three different virus stocks were obtained: rVV-594/605 contains both mutations, whereas in rVV-594 and rVV-605 both mutations are separated. All three recombinant viruses expressed pUL97 and the levels of UL97 protein expression were quantitatively similar (Fig. 2). However, after infection of TK-deficient 143B cells followed by HPLC analysis of the cell extracts, the three viruses exhibited different levels of GCV-phosphorylating activity. As shown in Fig. 3, in cells infected with the rVV-594/605 double mutant, the expected decrease in GCV phosphorylation could be observed, thus confirming the GCV resistance of the clinical HCMV isolate S.I. Compared with the GCV-phosphorylating activity measured in cells infected with the rVV-UL97WT, the activity was decreased to 50%. Interestingly, the rVVs with single mutations exhibited GCV-phosphorylating activities that were not identical to the activity of rVV-594/605. The single mutation carried by rVV-594 was associated with a further decrease of 20% in GCV phosphorylation compared with the double mutant, resulting in a residual activity of 30%. On the other hand, the mutation in rVV-605 led to an increase in the GCV-phosphorylating activity even more than that observed in cells infected with the rVV-UL97WT.

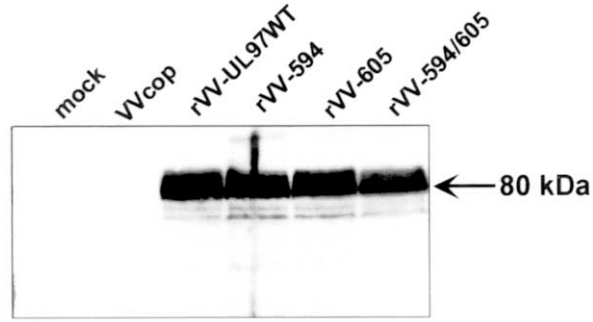

Fig. 2. Western blot analysis of rVV-infected cells. CV-1 cells were mock-infected or infected with the indicated vaccinia viruses and harvested at 24 h p.i. Total cell extracts were separated by SDS-PAGE. Proteins were visualized by using a specific UL97-antiserum and chemiluminiscence. The UL97 protein is marked by the black arrow. VVcop, vaccinia virus Copenhagen strain; rVV-UL97WT, recombinant vaccinia virus containing the UL97 coding region of the laboratory strain AD169.

3.4. Susceptibility of rVVs to GCV

The influence of the different mutations on the GCV susceptibility of the rVVs was evaluated by the plaque reduction test. As summarized in Fig. 4, the results of kinase assay were confirmed by the biological assay. The rVV double mutant was not as resistant as the rVV-594 (GCV IC_{50}: 690 and 820 μM, respectively). On the other hand, the D605E mutation conferred a higher GCV-sensitivity of the rVV-605 (GCV IC_{50}: 180 μM) as compared with the rVV-UL97WT (GCV IC_{50}: 280 μM).

4. Discussion

Sequence analysis of a GCV-resistant HCMV isolate from an AIDS patient revealed two point mutations in the UL97 gene. Both led to an

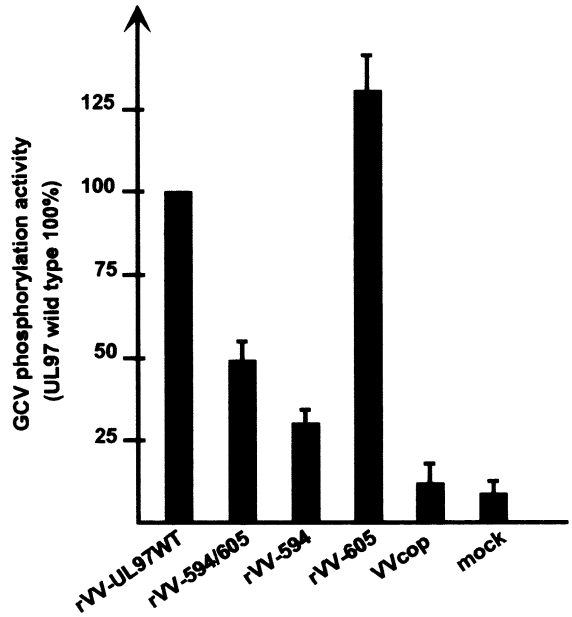

Fig. 3. GCV phosphorylation in 143B (TK$^-$) cells after mock-infection or infection with different vaccinia viruses. Cells were infected at an MOI of 10, harvested at 24 h p.i. and prepared for HPLC analysis. GCV phosphates were quantified by liquid scintillation counting. The bars represent the mean values from four independent experiments with standard deviations. VVcop, vaccinia virus Copenhagen strain.

amino acid substitution. The mutation at amino acid 594 corresponds to approximately 20% of that mutations observed in clinical GCV-resistant HCMV isolates. Although in most of these cases, either valine or threonine residue substituted for the alanine residue (reviewed by Erice, 1999), the replacement with proline has been never reported. Proline is special in that it may distort the backbone of polypeptide and cause the functional impairment of the UL97 protein. In most cases, GCV-resistant HCMV has a single mutation in UL97 gene and its impact on GCV resistance has been confirmed by marker transfer experiment. However, marker transfer experiments using the HCMV system are difficult and require selection pressure. We have attempted twice to transfer these two mutations into AD169 using the method described previously (Chou et al., 1995a), but did not succeed in isolating the recombinant HCMV. Although the precise reason of our failure is not clear, we used HEL cells, instead of MRC-5 fibroblasts, for transfection, which might cause failure. Metzger et al. (1994) have developed the rVV system to analyze the functional relevance of the UL97 gene mutation. The rVV system has an advantage over recombinant HCMV system in constructing recombinant virus. Therefore, using the rVV system and in vitro site mutagenesis, the attempt was made to determine the functional role of the individual mutations of S.I strain in relation to GCV resistance.

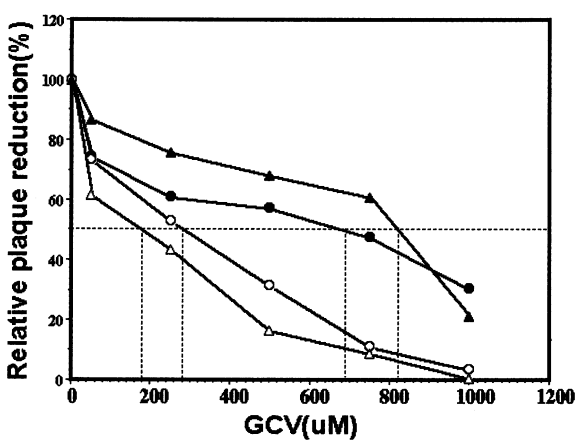

Fig. 4. Susceptibility of rVVs to GCV. rVV-UL97 (○–○); rVV-594/605 (●–●); rVV-594 (▲–▲); rVV-605 (△–△). See the text for the experimental conditions.

Using the rVV system it was shown that indeed both mutations have their functional relevance; namely, D605E mutation is lifting up the level of the decreased GCV phosphorylation due to the A594P mutation. This result was also confirmed by the higher GCV resistance of the rVV containing the A594P mutation than the rVV double mutant. On the other hand, the rVV containing the D605E mutation was more sensitive to GCV than the rVV-UL97WT. Therefore, the mutation at codon 605 might be inferred to compensate for the GCV resistance of the mutation at codon 594. To our knowledge, this is the first report showing the existence of two mutations, one conferring GCV resistance and another compensating for this resistance in the same HCMV genome. In HIV-1 it has been shown that suppression of resistance to drugs can occur during combination therapy if different mutations arise. The mutation M184V in the HIV-1 reverse transcriptase gene is

responsible for resistance to lamivudine. This mutation also arises during the treatment with didanosine and abacavir, and delays the development of phenotypical resistance towards zidovudine (Miller et al., 1998, 1999a,b). Furthermore, this mutation also increased the susceptibility to adefovir and tenofovir (Ait-Khaled, et al., 1999; Wainberg et al., 1999; Miller et al., 1999a,b). Apparently, the M184V mutation differentially affects the susceptibility to different antiviral drugs (Kuritzkes et al., 1996). In the case of HCMV, because the biological function of the UL97 protein is still unclear, it is presently too difficult to speculate about the influence of the mutation at codon 605 on the UL97 function.

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