Original Article

Acyclovir Sensitivity and Neurovirulence of Herpes Simplex Virus Type 1 with Amino Acid Substitutions in the Viral Thymidine Kinase Gene, Which Were Detected in the Patients with Intractable Herpes Simplex Encephalitis Previously Reported

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SUMMARY: Several cases of herpes simplex encephalitis (HSE) caused by acyclovir (ACV)-resistant herpes simplex virus type 1 (HSV-1) have been reported. Amino acid substitutions of R41H, Q125H, and A156V in the viral thymidine kinase (vTK) gene have been reported to confer ACV resistance. Recombinant HSV-1 clones, containing each amino acid substitution in the vTK gene, were generated using the bacterial artificial chromosome system. A recombinant HSV-1 with the Q125H substitution showed ACV resistance while the R41H or A156V substitutions were ACV-sensitive. Furthermore, the Q125H recombinant HSV-1 was less virulent than the repaired virus, but it maintained neurovirulence in mice at relatively high levels. Substitution of Q125H, which was detected in the neonatal HSE patient, conferred ACV resistance, but the substitutions of R41H and A156V, which were detected in immunocompetent adult HSE patients, did not. This suggests that HSE caused by ACV-resistant HSV-1 might be a very rare event to occur during the course of ACV treatment in immunocompetent patients. Showing resistance to ACV treatment does not always indicate emergence of ACV-resistant HSV-1 in HSE patients.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen that causes a wide spectrum of diseases, including encephalitis (herpes simplex encephalitis [HSE]). HSE, which occurs relatively frequently in adolescents and elderly individuals, is one of the most common sporadic viral encephalitis (1–3). HSE patients are treated with administration of acyclovir (ACV) (1–3).

ACV is a guanosine analogue molecule that is phosphorylated by the thymidine kinase (TK) of HSV-1 to form ACV monophosphate (4,5). Subsequently, ACV monophosphate is phosphorylated by cellular kinases to form ACV triphosphate (ACV-TP) (4,5). ACV-TP inhibits the viral DNA polymerase activities and is incorporated into the viral DNA, resulting in the termination of viral DNA elongation, showing the inhibitory effect on HSV-1 replication (6).

HSV-1 acquires ACV resistance due to mutations in the viral TK (vTK) gene or DNA polymerase gene, in which the former is far more common with an incidence of approximately 95% (5). ACV-resistant (ACVr) HSV-1 infections occur in immunocompromised patients, such as those who have undergone hematopoietic stem cell transplantation and those with congenital immunodeficiency (7–10).

Recently, there have been several reports describing ACVr HSV-1 as a demonstrated or suspected causative agent of intractable HSE (11–15). HSV-1, in which the 125th residue in the vTK (glutamine) was replaced with histidine (Q125H), appeared in the cerebrospinal fluid (CSF) of a neonate with HSV-1 encephalitis during the course of ACV treatment, suggesting that it might be ACVr (11). Schulte et al. and Bergmann et al., respectively, reported that the R41H and A156V amino acid substitutions in the vTK, which were detected in the CSF of immunocompetent adult HSE patients, might have conferred ACV resistance (12,13). These patients showed resistance to ACV treatment. As the emergence of ACVr HSV-1 in those studies were confirmed either by the indirect assay or sequence analysis, there is currently no convincing evidence that the R41H, Q125H, and A156V amino acid substitutions in the vTK confer ACV resistance (11–13).

To confirm whether these amino acid substitutions confer ACV resistance, viruses with each mutation in the same genetic background should be generated and tested for ACV sensitivities. This might be the only way to evaluate the impact of the mutations on ACV resistance.
In the present study, recombinant HSV-1 clones, which carried each of these amino acid substitutions, were generated using the bacterial artificial chromosome (BAC) system (17). The ACV sensitivity of each recombinant HSV-1 clone was measured using the plaque reduction assay (PRA), and the neurovirulence of the ACVr HSV-1 clone was determined in mice.

MATERIALS AND METHODS

Cells: Vero cells were cultured in Dulbecco’s Modified Eagle Essential Medium (DMEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 5% calf serum (CS, Thermo Fisher Scientific, Waltham, CA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific). COS7 cells were cultured under the same conditions, except 10% fetal bovine serum (FBS) was used in the culture medium (Biological Industries, Beit Haemek, Israel).

Viruses: Authentic HSV-1 strain F (HSV-1 F) (18) was a kind gift from Dr. Y. Kawaguchi at The University of Tokyo, Japan, under permission from Dr. B. Roizman at the University of Chicago, USA. ACVr HSV-1 strain TAR (HSV-1 TAR) (10,19), which was isolated from an immunocompromised patient, and recombinant HSV-1 clones generated from the whole genome of HSV-1 F cloned into a BAC (pYEbac102) (17) were propagated in cultured Vero cells in DMEM supplemented with 1% CS (DMEM-1CS). Cells infected with each HSV-1 clone were disrupted by sonication treatment on ice (once) or frozen and thawed (3 times) to obtain viral stocks. The viral stocks were obtained from the supernatant fractions of the medium after centrifugation and were stored at −80°C. The infectious dose of the viral stocks was determined using a standard plaque assay in Vero cells and was expressed as plaque forming units (pfu)/ml. The authentic HSV-1 F, had the nucleotide sequences of the vTK and viral DNA polymerase coding region, both of which were identical to the reference (GenBank accession number: GU734771.1). In addition, nucleotide sequences of those regions of the plasmid pYEbac102 used in this study and YK304—an HSV-1 clone generated from the pYEbac102 (17)—were identical to the reference (GU734771.1), with the exception of the nucleotide substitutions of C25T and C165G in the vTK coding region, which resulted in amino acid substitutions of H9Y and D55E, respectively.

Plasmids: To generate a plasmid pBS-delTK, the nucleotide residues 387–749 in the HSV-1 F TK gene were amplified by polymerase chain reaction (PCR) using the

| Objective | Sequence (5’ to 3’) |
|-----------|---------------------|
| Construction of pBS-delTK | GCCCTGAGCATGCTTATTGCGGAGGCC |
| Construction of pBS-delTK-KanR | GCCTGCTTCAAGAGACGCAAGACGTCGAGG |
| Generation of rHSV-1-R41H | TGGGATGACGATAGTGGG |
| Generation of rHSV-1-R41Hr | TGGGATGACGATAGTGGG |
| Generation of rHSV-1-Q125H | TGGGATGACGATAGTGGG |
| Generation of rHSV-1-Q125Hr | TGGGATGACGATAGTGGG |
| Generation of rHSV-1-A156V | TGGGATGACGATAGTGGG |
| Generation of rHSV-1-A156Vr | TGGGATGACGATAGTGGG |
primer sets (Table 1) and cloned into the XhoI and KpnI sites of pBluescript II KS (+) (Stratagene, La Jolla, CA, USA).

To generate a plasmid pBS-delTK-KanR, the I-SceI restriction site and aphA1 gene of pEP-KanS (20)—kindly provided by Dr. Y. Kawaguchi under permission from Dr. N. Osterrieder at Freie Universität Berlin, Germany—were amplified by PCR using the primer sets (Table 1) and cloned into the PspOMI site of pBS-delTK.

Construction of recombinant HSV-1: The recombinant viruses, rHSV-1-R41H, rHSV-1-Q125H, and rHSV-1-A156V, carrying the R41H, Q125H, and A156V amino acid substitutions in their vTK, respectively, were constructed by the 2-step Red-mediated mutagenesis procedure using Escherichia coli GS1783 containing pYEbac102, a full-length infectious HSV-1 F clone (17)—a gift from Dr. Y. Kawaguchi, with permission for use from Dr. G.A. Smith of Northwestern University, USA and Dr. N. Osterrieder—as described previously using the primer sets (Table 1, Fig. 1) (20,21).

The recombinant viruses, rHSV-1-R41Hr and rHSV-1-A156Vr, in which the R41H and the A156V amino acid substitutions in rHSV-1-R41H and rHSV-1-A156V were repaired, respectively, were constructed as described previously using the primer sets (Table 1, Fig. 1) (20,21).

The vTK activity-deficient recombinant virus, rHSV-1-delTK, in which the nucleotide residues 387–749 in the vTK gene of rHSV-1-Q125H were deleted according to the previous study (22) and the Q125H substitution was repaired, was constructed as described previously using the primer sets (Table 1, Fig. 1) (20,21).

The recombinant virus rHSV-1-Q125Hr, in which the deleted nucleotide residues of the vTK gene in rHSV-1-delTK were repaired, was constructed as described previously using a DNA fragment, which was PCR amplified from pBS-delTK-KanR using the primer set (Table 1, Fig. 1) (20,21,23).

To generate recombinant viruses, COS7 cells were transfected with each BAC containing the full-genome of the recombinant HSV-1 using the FuGENE6 transfection reagent (Promega, Madison, WI, USA).

In vitro viral replication: Vero cells were inoculated in triplicate with each virus at a multiplicity of infection (moi) of 5 per cell and incubated for 1 h at 37°C under 5% CO2. Subsequently, the cells were washed 3 times with phosphate buffered saline solution (PBS) and cultured in DMEM-1CS. The cell cultures were harvested at designated time points, and the viral infectious doses were measured by a standard plaque assay in Vero cells.

Western blotting: Vero cells were inoculated with mock inoculum or each virus at an moi of 1 per cell, harvested 24 h after inoculation, and subjected to Western blotting as described previously with some modifications (19,24). Rabbit anti-HSV-1 TK antibody (19) and mouse anti-HSV-1 and herpes simplex virus type 2 ICP5 antibody (Abcam, Cambridge, UK), which were diluted 1,000 times in T-PBS (PBS with 0.1% Polyoxyethylene Sorbitan Monolaurate [Wako Pure Chemical Industries]), were used as the primary antibodies to detect vTK and ICP5 polypeptide expression. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG antibody (SeraCare

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Fig. 1. Schematic diagrams of the generation of recombinant HSV-1 with amino acid substitution and the repairs. The full-length genome of YK304—an HSV-1 clone generated from the pYEbac102 (17)—in the linear form is presented at the top. The only vTK gene (UL23) of each recombinant virus is depicted in the figure.
Life Sciences, Milford, MA, USA) and HRP-labeled goat anti-mouse IgG antibody (SeraCare Life Sciences), which were diluted 5,000 times and 3,000 times in T-PBS, respectively, were used as the secondary antibodies for detection of the vTK and ICP5 polypeptides, respectively.

Sensitivity of viruses to ACV and other vTK-associated compounds: The sensitivities of the viruses to antiviral compounds, including ACV (Tokyo Chemical Industry, Tokyo, Japan), ganciclovir (GCV; Tokyo Chemical Industry), and penciclovir (PCV; Tokyo Chemical Industry), were evaluated in Vero cells with a PRA as described previously (10,19,24–27). The 50% inhibitory concentration (IC50) of each antiviral compound to each virus was calculated graphically.

Neurovirulence of rHSV-1-Q125H in mice: The neurovirulence of the viruses was measured as described previously (25). In brief, 3-week-old female ICR mice (Japan SLC, Inc., Shizuoka, Japan) were intracerebrally inoculated with the designated infectious doses of rHSV-1-Q125H, rHSV-1-delTK, or rHSV-1-Q125Hr in 50 µl of DMEM under anesthesia using a combination of medetomidine, midazolam, and butorphanol. Three mice were inoculated with the viral solution at each dilution level. Survival and the body weight of the mice were monitored daily for 14 days after inoculation, and the 50% lethal dose (LD50) of each virus was calculated by the Reed and Muench method. When the mice lost more than 20% of their initial body weight, they were euthanized by exposure to excess CO2. The experiments were conducted twice, independently.

Ethical statement: The animal experiments were conducted in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan and in strict compliance with animal husbandry and welfare regulations under the approval of Animal Care and Use Committee of the National Institute of Infectious Diseases (No. 117026).

RESULTS

Generation of recombinant HSV-1 clones with the amino acid substitutions in their vTK: The recombinant viruses did not have any additional or unintended changes in their vTK or viral DNA polymerase genes from pYebac102 and YK304. The 2 amino acid substitutions, H9Y and D55E, in the vTK polypeptide of all recombinant viruses did not affect the phenotypes of these recombinant viruses with regard to the replication kinetics, the expression of vTK polypeptide in Vero cells, and ACV, GCV, and PCV sensitivities (Fig. 2, Fig. 3, and Table 2).

Growth kinetics and the vTK expression of recombinant HSV-1 clones in Vero cells: All recombinant HSV-1 clones replicated with the same growth capacity as HSV-1 F (Fig. 2). With the exception of rHSV-1-delTK, the vTK polypeptide expression levels of the recombinant viruses were in line with that of the authentic HSV-1 F (Fig. 3). In contrast with the vTK shown in the other lanes, rHSV-1-delTK showed a blurred band between 32 kDa and 27 kDa (Fig. 3).

Sensitivities of rHSV-1-R41H, rHSV-1-Q125H, rHSV-1-A156V, and their revertant viruses to vTK-associated antiviral compounds: The IC50 values of ACV, GCV, and PCV to each recombinant HSV-1 clone are shown (Table 2). The IC50 values of these antiviral compounds to rHSV-1-R41Hr, rHSV-1-Q125Hr, and rHSV-1-A156Vr were similar to those of HSV-1 F, indicating the 2 amino acid substitutions, H9Y and D55E in the vTK of all recombinant HSV-1 clones, did not confer ACV, GCV, and PCV resistance. rHSV-1-Q125H showed resistance not only to ACV, but also to GCV and PCV.

Neurovirulence of rHSV-1-Q125H in mice: The neurovirulence of rHSV-1-Q125H, rHSV-1-delTK, and...
ACV Sensitivity and Neurovirulence of HSV-1 rHSV-1-Q125Hr in mice was determined by inoculating intracerebrally with each of the recombinant viruses. The LD₅₀ values of rHSV-1-Q125H, rHSV-1-delTK, and rHSV-1-Q125Hr in Experiment 1 were 10².50, 10⁴.25, and 10¹.75 pfu, respectively, while those in Experiment 2 were 10².75, 10⁴.25, and 10¹.75 pfu, respectively.

**DISCUSSION**

The novelty of this study was that recombinant HSV-1 clones expressing amino acid substitutions, which were suspected of conferring ACV resistance in HSE patients, were generated using the BAC system and tested for ACV sensitivity and neurovirulence in mice.

All recombinant viruses carrying each of the vTK mutations showed the same growth property as their repaired counterparts and wild-type HSV-1 F (Fig. 2), as the vTK gene of HSV-1 is dispensable for viral replication in vitro (25,28). As such, probable truncated vTK polypeptide shown as a blurred band between 32 kDa and 27 kDa was expressed in the cells infected with rHSV-1-delTK (Fig. 3). These results indicate that recombinant viruses with each vTK mutation in the same genetic background were generated successfully.

To the best of our knowledge, there are no case reports describing the isolation of infectious HSV-1 from the CSF of HSE patients, showing that the isolation of infectious HSV-1 is quite difficult (2). The difficulty might be due to the presence of neutralizing antibodies in the CSF (1,13), even though HSV-1 replicates in the central nervous system (CNS) (11,13). Thus, this difficulty makes it impossible to directly evaluate the sensitivities of the causative HSV-1 to ACV and other antiviral agents. In previous reports, the nucleotide sequences of the vTK genes PCR-amplified from CSF of HSE patients were determined to speculate whether the causative HSV-1 was ACVr based on previously reported ACV resistance-associated substitutions (11–15).

The patient information described in these reports is summarized in Table 3. Two HSE cases were definitively caused by ACVr HSV-1 because frameshift mutations were detected in the vTK genes amplified from CSF samples collected from a patient with chronic lymphocytic leukemia (CLL) and from another patient.

![Fig. 3. The expression of vTK and ICP5 polypeptides of recombinant HSV-1 clones. Vero cells were infected with each virus at an moi of 1 per cell and harvested at 24 hours after infection for Western blotting. ICP5 was set as the control. The white arrowhead indicates the probable truncated vTK polypeptide.](image)

Table 2. The 50% inhibitory concentration values of the antiviral compounds on each HSV-1 clone

| Antiviral compounds | HSV-1 F | rHSV-1-Q125H | rHSV-1-delTK | rHSV-1-Q125Hr | rHSV-1-R41H | rHSV-1-R41Hr | rHSV-1-A156V | rHSV-1-A156Vr | HSV-1 TAR |
|---------------------|---------|--------------|-------------|--------------|-------------|-------------|-------------|-------------|----------|
| ACV**₁**            | 0.64 ± 0.07 | 8.5 ± 1.64  | 0.51 ± 0.01 | 0.52 ± 0.02  | 0.51 ± 0.02 | 0.59 ± 0.02 | 0.49 ± 0.01 | ≥40         |
| GCV**₁**            | 0.21    | 2.5          | 6.8         | 0.14         | NT          | NT          | NT          | NT          |
| PCV**₁**            | 1.2     | 6.3          | 24          | 0.79         | NT          | NT          | NT          | NT          |

*₁: The data indicate the mean ± standard deviation from 2 independent experiments.
*₂: The data are representative of 3 independent experiments.
NT: not tested.
receiving anti-tumor necrosis factor (TNF)-alpha monoclonal antibodies (14,15).

The Q125H amino acid substitution in the vTK conferred ACV resistance, consistent with the previous study (11). In the neonatal HSE case, the HSV-1 genome containing the Q125H amino acid substitution in the vTK increased during the course of ACV therapy (11). Therefore, the virus, was definitely ACVr HSV-1.

The other 2 amino acid substitutions, R41H and A156V, did not confer ACV resistance. HSV-1 clones with R41H and A156V amino acid substitutions in the vTK were reported to be ACV-sensitive in previous reports (7,29–31). Brunemann and colleagues generated recombinant viruses, which carried any one of 11 amino acid substitutions including R41H, and showed that the R41H amino acid substitution did not confer ACV resistance (29). This study supports the results that the R41H amino acid substitution did not confer ACV resistance (29). Therefore, there is no evidence that ACVr HSV-1 had emerged in the reported immunocompetent adult HSE patients (12,13). So far, the reports describing the emergence of ACVr HSV-1 in the CNS of HSE patients are restricted to the cases of the neonate and immunocompromised patients (11,14,15).

vTK-deficient rHSV-1-delTK, in which nucleotide residues 387–749 in the vTK gene were deleted according to the previous study (22), lacked the expression of functional vTK (Fig. 3). It was also expected to reduce expression of the UL24 gene (34,35). It was reported that the impairment of UL24 polypeptide reduced viral replication capacity in vitro and in the mouse trigeminal ganglia (28,36). However, the replication capacity of rHSV-1-delTK was in line with other viruses, including rHSV-1-Q125Hr and HSV-1 F (Fig. 2). Although it was unclear whether reduction of UL24 polypeptide expression in rHSV-1-delTK affected its attenuation, the attenuation of rHSV-1-delTK to neurovirulence was of the same magnitude as the vTK-frameshifted and -deficient HSV-1 (25,32,33).

In summary, these results suggest that the HSE, which shows resistance to ACV treatment, differs from the HSE caused by ACVr HSV-1. HSE caused by ACVr HSV-1 might occur in neonates and immunocompromised patients. The emergence of ACVr HSV-1 in the CNS of immunocompetent adult HSE patients might be a rare event.

Acknowledgments We appreciate Dr. Yasushi Kawaguchi, who kindly provided us with HSV-1 F, E. coli GS1783 containing pYEBac102, and a plasmid pEP-kanS, and we also appreciate Dr. Bernard Roizman, Dr. Gregory A. Smith, and Dr. Nikolaus Osterrieder to give the permission for it. We thank Ms. Yoshiko Fukui and Ms. Mihoko Tsuda for their assistance in this work. This work was financially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Nos. 15K09675, 24591591, and 18K07894), and a grant from the Japan Foundation for Pediatric Research (Grant No. 14-015).

Conflict of interest None to declare.

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