Original Article

New Mechanism of Acyclovir Resistance in Herpes Simplex Virus 1, Which Has a UAG Stop Codon between the First and Second AUG Initiation Codons

Phu Hoang Anh Nguyen^{1,2}, Souichi Yamada¹, Miho Shibamura¹, Takuya Inagaki^{1,3}, Hikaru Fujii^{1,4}, Shizuko Harada¹, Shuetsu Fukushi¹, Masashi Mizuguchi², and Masayuki Saijo^{1,2*}

¹*Department of Virology 1, National Institute of Infectious Diseases, Tokyo ;*

²*Department of Developmental Medical Sciences, The University of Tokyo, Tokyo ;*

³*Department of Life Science and Medical Bioscience, Waseda University, Tokyo ; and*

⁴*Department of Microbiology, The Faculty of Veterinary Medicine, Okayama University of Science, Ehime, Japan*

SUMMARY: Morphological changes in the structure of the herpes simplex virus 1 (HSV-1) viral thymidine kinase (vTK) polypeptide usually lead to conferring acyclovir (ACV) resistance. HSV-1 I4-2, in which a UAG stop codon is present at the 8th position between the 1st initiation AUG codon (1st position) and the 2nd initiation AUG codon (46th position) of the HSV-1 vTK gene, showed sensitivity to ACV. In contrast, HSV-1 KG111, in which a UAG stop codon was artificially inserted at the 44th position, showed resistance to ACV at 39°C. The mechanism underlying the difference in the sensitivity profiles was elucidated. The virus recombinants HSV-1-TK(8UAG) and HSV-1-TK(44UAG) containing a UAG stop codon at the 8th and 44th positions counted from the 1st initiation codon, respectively, were generated and tested for susceptibility to antiviral compounds. HSV-1-TK(8UAG) and HSV-1-TK(44UAG) were sensitive and resistant to ACV and BVdU at 37°C, respectively. The expression level of the truncated vTK translated from the 2nd initiation codon in Vero cells infected with HSV-1-TK(44UAG) was clearly less than that with HSV-1-TK(8UAG) in a temperature-dependent manner. The differences in the antiviral sensitivity profiles were due to the position of the UAG stop codon between the 1st and the 2nd initiation codons.

INTRODUCTION

The treatment of choice for alphaherpesvirus (herpes simplex virus 1 [HSV-1], herpes simplex virus 2, and varicella-zoster virus) infections is 9-(2-Hydroxyethoxy)-methylguanine (acyclovir [ACV]). The viral thymidine kinase (vTK) expressed by these viruses phosphorylates ACV *in vitro* and *in vivo* to form ACV-monophosphate (ACV-MP) (1–3). ACV-MP is then phosphorylated by cellular kinases to form ACV-triphosphate (ACV-TP), the active form, through a phosphorylation step that forms ACV-diphosphate (ACV-DP) (4,5). (E)-5-(2-Bromovinyl)-2'-deoxyuridine (BVdU) also inhibits the replication of HSV-1 by a similar mechanism of action to ACV. HSV-1 vTK phosphorylates BVdU to BVdU-DP by forming BVdU-MP. The host kinases subsequently

phosphorylate BVdU-DP to the active form BVdU-TP (5). Therefore, the phosphorylation activity of HSV-1 vTK for vTK-associated compounds such as ACV and BVdU is a critical factor involved in the sensitivity of HSV-1 to ACV and other vTK-associated antiherpetic compounds.

An HSV-1 isolate, HSV-1 I4-2, which encodes a UAG stop codon at the 8th position counted from the 1st AUG initiation (1st AUG) codon, is circulating in the general population (6). The HSV-1 I4-2 isolate does not express the 43 kD-sized HSV-1 vTK polypeptide composed of 376 amino acid residues translated from the 1st AUG codon but expresses a 39 kD-sized HSV-1 vTK polypeptide consisting of 331 amino acids translated from the 2nd AUG codon at the 46th position (7). The HSV-1 vTK expressed by HSV-1 I4-2 lacks the 45 amino acid residues of the amino-terminus. HSV-1 I4-2 is sensitive to ACV at both 37°C and 39°C (7).

The HSV-1 KG111, which was generated artificially and in which a UAG stop codon was inserted at the 44th position, was reported to express the 39 kD-sized HSV-1 vTK translated from the 2nd AUG codon and to be ACV resistant at a temperature of 39°C but ACV sensitive at a temperature of 34°C, suggesting that the expression of the truncated vTK expressed by HSV-1 KG111 was thermo-unstable (8).

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*Corresponding author: Mailing address: Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan. Tel: +81-3-4582-2660, Fax: +81-3-5285-1150, E-mail: msaijo@nih.go.jp

There is a contradiction in terms of the sensitivity profiles to ACV and BVdU between HSV-1 I4-2 and HSV-1 KG111. Both HSV-1 I4-2 and HSV-1 KG111 have a UAG stop codon between the 1st AUG and the 2nd AUG codons, but the position of the UAG codon is different, being that the UAG codons of the former and the latter are at the 8th and the 44th positions, respectively, as counted from the 1st AUG initiation codon.

It is speculated that the mechanism for the difference in the antiviral sensitivity profiles depends on the position of the UAG codon. The present study aimed to confirm that HSV-1, whose vTK gene has a UAG stop codon between the 1st AUG and 2nd AUG initiation codons, is sensitive or resistant to ACV and BVdU in association with the effect of temperature. Furthermore, the effect of the positions of the UAG stop codon on the expression levels of vTK was evaluated. From these studies, a new mechanism for acquiring ACV resistance was elucidated.

MATERIALS AND METHODS

Cells: Human embryonic lung fibroblast (HELFL), Vero cells (African green monkey kidney-derived cell line), and COS-7 cells (CV-1 African green monkey kidney fibroblast-like cell lines) were used. HELFL cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Beit Haemek, Israel) and penicillin-G (100 U/mL) and streptomycin (100 µg/mL) (MEM-10FBS). Vero cells were cultured in Dulbecco's modified Eagle essential medium containing penicillin-G (100 U/mL), streptomycin (100 µg/mL), and 5% calf serum (Thermo Fisher Scientific, Waltham, MA, USA) (DMEM-5CS) (9). The HELFL cells and Vero cells were used for testing antiviral susceptibility (10). COS-7 cells were cultured in DMEM with similar antibiotic supplements and 10% calf serum (DMEM-10CS), and COS-7 cells were used for recombinant virus reconstitution (9,11).

Viruses: HSV-1 TAR strain, which was isolated from a child with Wiskott-Aldrich syndrome and resistant to ACV due to the frameshift mutation in the viral TK gene, was used as a control of ACV-resistant HSV-1 (6,11). HSV-1 strain F provided by Dr. Y. Kawaguchi at The University of Tokyo, Japan, under permission from Dr. B. Roizman at the University of Chicago, IL, was used as a positive control for the detection of HSV-1 vTK with a Western blotting assay.

Compounds: The ACV and BVdU used were purchased from Tokyo Chemical Industry (Tokyo, Japan).

Construction of recombinant HSV-1: The recombinant HSV-1 strains HSV-1-TK(8UAG) and HSV-1-TK(44UAG) were generated using a two-step Red recombination system, as described previously (9,10,12–14) with some modifications. The recombinant HSV-1 WT was also generated as the control virus. *Escherichia coli* GS1783 containing pYEbac102 that carried the parental HSV-1 F strain and pEP-KanS, which was the shuttle vector containing a kanamycin resistance cassette, was used for BAC mutagenesis to generate the HSV-1 recombinant HSV-1 mutants. HSV-

1 WT without any mutations was also constructed as the ACV-sensitive control. For the construction of recombinant HSV-1-TK(8UAG), the following primer set was used: forward primer 5'-GCGCCTTGTAGAA GCGCGTATGGCTTCGTACCCCTGCCATTAGTAC GCGTCTGCGTTTCGACCAAGGATGACGACGATA AGTAGGG-3' (UAG mutation site is underlined) and reverse primer 5'-GGCCGCGAGAACGCGCAGCC TGGTCGAACGCAGACGCGTACTAATGGCAGG GGTACGAAGCCACAACCAATTAACCAATTCTG ATTAG-3' (UAG mutation site is underlined). For the construction of recombinant HSV-1-TK(44UAG), the following primer set was used: forward primer 5'-CCC TCGCCGGCAGCAAGAAGCCACGGAAGTCCGCC TGGAGTAGAAAATGCCACGCTACTGCGAGGAT GACGACGATAAGTAGGG-3' (UAG mutation site is underlined) and reverse primer 5'-GAGGACCCTCTAT ATAAACCCGCAGTAGCGTGGGCATTTTCTACTC CAGGCGGACTTCCGTGGCAACCAATTAACCAAT TCTGATTAG-3' (UAG mutation site is underlined).

The vTK gene nucleotide sequence of each generated recombinant virus was confirmed to have the intended sequence but not to have any additional and unintended sequences as that of pYEbac102.

The inhibitory effect of ACV and BVdU against the replication of these recombinant HSV-1 mutants was also evaluated by plaque reduction assay as reported previously with some modifications such as the temperature conditions (6,7,9). The inhibitory effect of ACV and BVdU on the viruses was evaluated in Vero cell monolayers and HELFL cell monolayers, respectively. Cell monolayers cultured in 24 well-culture plates were inoculated with each recombinant HSV-1 mutant as to be approximately 50 plaque-forming units (PFU) per well. The concentrations of ACV tested were 0, 0.04, 0.4, 1, 4, and 10 µg/mL, whereas those of BVdU were 0, 0.01, 0.1, 1, and 10 µg/mL.

Susceptibility test with yield reduction assay: The inhibitory effect of ACV on the replication of HSV-1 recombinants was evaluated on HELFL cells by a yield reduction assay at 37°C as reported previously with some modifications (7). Briefly, HELFL cells as a form of monolayer cultured were inoculated with each virus at a multiplicity of infection (MOI) of 0.1 PFU per cell and incubated at 37°C for 1 h. After absorption, the inocula were removed, and the cells were washed with phosphate-buffered saline solution (PBS). The cells were then fed with MEM containing 2% of FBS (MEM-2FBS) and designated concentrations of ACV (0, 0.4, 1, 4, and 10 µg/mL). After an incubation period of 24 h at 37°C, the cultures were frozen and thawed 3 times. The medium was then centrifuged at 3,000 rpm for 5 min at 4°C, and the virus titers in the supernatants were determined in Vero cells by plaque assay.

Western blotting: TK expression of vTK polypeptides in the cells infected with recombinant HSV-1 WT, HSV-1-TK(8UAG), HSV-1-TK(44UAG), and mock cultured at a temperature of either 35°C or 37°C was confirmed as reported previously (6,7). Briefly, Vero cells were infected with each HSV-1 recombinant at a MOI of 3. After 1 h of absorption, the virus solution was removed and washed once with PBS to discard unabsorbed viruses and then fed with DMEM-2CS and incubated at a temperature of either

Fig. 1. The vTK nucleotide sequence of HSV-1 WT, HSV-1-8TK, and HSV-1-44TK. The number indicates the position of the codon counted from the 1st AUG initiation codon. The UAG stop codon artificially inserted is underlined. The 3-nucleotide codon in bold indicates the 1st and the 2nd AUG initiation codons at the 1st and 46th positions counted from the 1st initiation codon.

35°C or 37°C under humidified conditions with 5% CO₂ for 24 h. The cells were then harvested, washed once with PBS, treated with lysis buffer (50 mM Tris-HCl [pH 8.0] [Merck, Darmstadt, Germany], 150 mM NaCl [Fujifilm Wako Pure Chemical, Osaka, Japan], 1% Nonidet P-40 [Nacalai Tesque, Kyoto, Japan], and Complete Mini [Roche, Basel, Switzerland]) for 20 min at 4°C and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatants were collected and subjected to Western blotting. For the detection of HSV-1 vTK polypeptide, a rabbit serum raised against recombinant HSV-1 TK was used (6). The expression of HSV-1 envelope glycoprotein B (gB) (Abcam, Cambridge, UK) was also tested for expression by Western blotting as an internal control.

Statistical analysis: The differences in the sensitivity of each of the recombinant HSV-1s to ACV and BVdU were statistically evaluated by a Student *t*-test after transforming the IC₅₀ values to Log₁₀ values. A *p*-value < 0.05 was considered to indicate statistical significance.

RESULTS

Construction of recombinant HSV-1: Recombinant HSV-1 WT was generated using the pYEbac102 BAC system. It possessed 2 nucleotide substitutions of C25T and C165G in the vTK coding region, leading to a change of 2 amino acid substitutions H9Y and D55E compared to the original HSV-1 F strain (GenBank accession number: GU734771.1), as reported previously (9). However, it was confirmed that these 2 amino acid substitutions did not affect the replication capacity and sensitivities to ACV and several vTK-associated antiherpetic compounds or the expression of vTK (9). Recombinant HSV-1-TK(8UAG) and HSV-1-TK(44UAG) were successfully constructed. The vTK gene nucleotide sequences of HSV-1-TK(8UAG) and

HSV-1-TK(44UAG) were confirmed to be identical to that of the parental HSV-1 WT except for the UAG stop codon at the 8th position and 44th position, respectively (Fig. 1).

Sensitivity to antiviral compounds of HSV-1 evaluated with plaque reduction assay: The IC₅₀ values of ACV to HSV-1 WT, HSV-1-TK(8UAG), HSV-1-TK(44UAG), and TAR were 0.71 ± 0.11 µg/mL, 1.4 ± 0.06 µg/mL, 5.13 ± 0.23 µg/mL, and > 10 µg/mL, respectively, whereas those of BVdU were 0.25 ± 0.02 µg/mL, 0.4 ± 0.05 µg/mL, > 10 µg/mL, and > 10 µg/mL, respectively (Fig. 2A). HSV-1 WT was the most sensitive to ACV among the recombinant viruses. HSV-1-TK(44UAG) was more resistant to ACV and BVdU than was HSV-1-TK(8UAG). HSV-1-TK(8UAG) showed less sensitivity to BVdU than HSV-1 WT. HSV-1-TK(44UAG) was highly resistant to BVdU with the IC₅₀ value of > 10 µg/mL as with HSV-1 TAR (Fig. 2A). As expected, ACV and BVdU had no inhibitory effect on the replication of HSV-1 TAR. The differences in IC₅₀ values of ACV and BVdU were statistically significant among the recombinant viruses HSV-1 WT, HSV-1-TK(8UAG), and HSV-1-TK(44UAG).

Sensitivity to antiviral compounds of the recombinant HSV-1 evaluated with yield reduction assay: HSV-1-TK(44UAG) was confirmed to be more resistant to antiviral compounds than HSV-1 WT and HSV-1-TK(8UAG) (Fig. 2B). The sensitivity patterns of these recombinant viruses to ACV determined with the yield reduction assay were the same as those determined with the plaque reduction assay.

Expression of vTK at 35°C and 37°C in the virus-infected Vero cells: Because the temperature was expected to influence HSV-1 vTK polypeptide expression as reported previously (7), the HSV-1 vTK polypeptide expression levels in cells cultured at the temperatures of 35°C and 37°C were evaluated with

Fig. 2. (Color online) Inhibitory effects of ACV and BVdU on the replication of HSV-1 WT, HSV-1-TK(8UAG), HSV-1-TK(44UAG), and HSV-1 TAR determined with plaque reduction assay (A) and those of ACV determined with the yield reduction assay (B). The replication curves of each virus in the presence of a designated concentration of ACV in Vero cells cultured at 37°C (A, upper panel) and those to BVdU in HELF cells cultured at 37°C (A, lower panel) are shown. The number of plaques formed by each virus at the lowest concentration was almost identical to those of mock (control). The experiments were independently repeated 3 times. The results are shown as the average of 3 experiments with standard deviations. The inhibitory effects of ACV on the replication of HSV-1 WT, HSV-1-8TK, HSV-1-44TK, and TAR determined in HELF cells cultured at a temperature of 37°C with the yield reduction assay are shown (B).

Western blotting analyses (Fig. 3). The molecular weight of the vTK polypeptide expressed in HSV-1-TK(8UAG)- or HSV-1-TK(44UAG)-infected Vero cells was expected to be approximately 39 kD, whereas that of the HSV-1 WT- or HSV-1 F-infected Vero cells was 43 kD. The 43 kD-vTK polypeptide was expressed similarly in the HSV-1 WT- or HSV-1 F-infected Vero cells when the virus-infected cells were cultured at both 35°C and 37°C (Fig. 3). The 39 kD-HSV-1 vTK polypeptide was expressed in the HSV-1-TK(8UAG)-infected Vero cells, but the expression level at 37°C was lower than that at 35°C (Fig. 3). On the contrary, the expression of the 39 kD-HSV-1 vTK polypeptide was not visualized in Western blotting in the HSV-1-TK(44UAG)-infected Vero cells at either 35°C or 37°C (Fig. 3). By making the visualization level highly sensitive, a very weak signal of the 39 kD-HSV-1 vTK polypeptide was detected in the lane of HSV-1-TK(44UAG)-infected Vero cells cultured at 35°C but not in the lane for cells cultured at 37°C (data not shown). Interestingly, in the Western blot analyses, the intensities of the vTK polypeptides expressed in the HSV-1-TK(8UAG)-infected Vero cells were weaker than those in the HSV-1 WT-infected cells at both culture conditions of 35°C and 37°C (Fig. 3). Furthermore, the intensities of the HSV-1 vTK polypeptide expressed by Vero cells infected with HSV-1-TK(8UAG) cultured at 37°C were weaker than those in cells cultured at 35°C. These results show that the expression levels of vTK polypeptides in both the HSV-1-TK(8UAG)-infected Vero cells and the HSV-1-TK(44UAG)-infected Vero cells were significantly reduced when cultured at 37°C.

DISCUSSION

Fig. 3. Expression of the vTK polypeptides in Vero cells infected with HSV-1 WT, HSV-1-8TK, HSV-1-44TK, or HSV-1 F cultured at the temperatures of 35°C and 37°C by Western blot analysis. The molecular weight of the proteins is shown in the left margin. Vero cells were infected with the indicated HSV-1 recombinants or mock. HSV-1 F indicates the vTK positive control.

It was reported that HSV-1 I4-2 showed phenotypically similar antiviral sensitivities to ACV and other vTK-associated antiherpetic drugs such as BVdU, 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BVaraU), and 1-beta-D-arabinofuranosylthymine (araT) as those of wild-type HSV-1 TAS without this mutation (7). Besides the TK activities, vTK possesses TMP-kinase that is required for the phosphorylation of

BVdU into BVdU-DP by forming BVdU-MP. The host kinase subsequently phosphorylates BVdU-DP into the active form, BVdU-TP (5,15). The sensitivity profiles of the viruses studied in the present and the previous studies indicate that the truncated vTK translated from the 2nd initiation codon possesses phosphorylation activity not only of BVdU to form BVdU-MP but also to phosphorylate BVdU-MP to form BVdU-DP (7).

HSV-1-TK(44UAG), an HSV-1 KG111-type virus, showed resistance to ACV and BVdU, whereas HSV-1-TK(8UAG), an HSV-1 I4-2-type virus, showed ACV sensitivity, although the IC₅₀ values of ACV and BVdU were relatively higher than those of HSV-1 WT. The same evidence was also confirmed by performing a yield reduction assay (Fig. 2). These results appear to verify the phenomenon that the HSV-1 KG111-type virus is resistant to ACV and BVdU, whereas the HSV-1 I4-2-type virus is sensitive to these compounds at 37°C. The only difference in the virus genome between HSV-1-TK(8UAG) and HSV-1-TK(44UAG) is the position of the UAG codon, indicating that the position of this UAG codon between the 1st and the 2nd AUG initiation codons is responsible for the difference in the sensitivity profiles.

The mechanism of HSV-1-TK(44UAG) acquiring resistance to ACV and BVdU was explored by measuring the 39-kD vTK expression in the cells infected with each recombinant virus with Western blotting analysis (Fig. 3). Interestingly, the HSV-1 vTK expression level in the cells infected with HSV-1-TK(44UAG) cultured at 35°C was clearly weaker than that in the cells infected with HSV-1-TK(8UAG) cultured at the same temperature. Furthermore, the HSV-1 vTK expression levels in the cells infected with HSV-1-TK(44UAG) or HSV-1-TK(8UAG) cultured at 37°C were lower than those in the cells cultured at 35°C. These results indicate that the HSV-1 vTK polypeptide expression levels of HSV-1, which has a UAG stop codon between the 1st and the 2nd AUG initiation codon in the vTK gene, is dependent on the position.

In conclusion, the difference in the antiviral sensitivity profiles between HSV-1 I4-2 (6,7) and HSV-1 KG111 (8) solely depended on the difference in the vTK expression levels in a temperature-dependent manner due to the locations of the UAG stop codon between the 1st and the 2nd AUG initiation codons.

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Conflict of interest None to declare.

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