Emergence of resistance to carbocyclic oxetanocin G in herpes simplex virus type 1 and genetic analysis of resistant mutants

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KEY WORDS carbocyclic oxetanocin G; human herpesvirus 1; viral drug resistance; thymidine kinase

ABSTRACT

AIM: To elucidate the potentiality of emergence of drug-resistance to carbocyclic oxetanocin G (C.OXT-G), a new effective antiviral drug for herpetic keratitis during treatment and the mechanism of this drug resistance. **METHODS:** A C.OXT-G resistant strain (C.OXT-G^r) was established by serially propagating the herpes simplex virus (HSV) -1 in African green monkey kidney (VERO) cells in the presence of C.OXT-G. After the drug sensitivity assay and the thymidine kinase (TK) activity assay, the molecular basis for the drug resistance was studied using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and PCR direct sequencing technology. **RESULTS:** After the 10th passage in 10 μm C.OXT-G, the ED_{50} of the C.OXT-G^r was 17.08-fold greater than that of the original strain on the average and the TK activities of these resistant strains were extremely reduced. PCR-SSCP analysis on TK gene of the wild HSV-1 and the C.OXT-G^r showed altered migration patterns in part 3 and part 4, while PCR-SSCP analysis on DNA polymerase gene showed no difference among the viruses. Sequence analysis revealed a deletion of G at position of 430 that caused frameshift, resulting in premature termination in the TK gene. **CONCLUSION:** The drug resistance to C.OXT-G may appear during the treatment due to the deficiency of TK activity caused by a single mutation in the TK gene of HSV-1.

INTRODUCTION

Carbocyclic oxetanocin G (C.OXT-G) was a new antiviral compound which is active against not only HSV but also other virus including varicella zoster virus, cytomegalovirus, Epstein Barr virus, and human immunodeficiency virus[1-3]. It showed an excellent therapetic effect in the treatment of herpetic keratitis[4,5]. C.OXT-G was phosphorylated by thymidine kinase (TK) to C.OXT-G monophosphate and finally to its triphosphate form, which might inhibit DNA polymerase of the virus[6-8]. One concern about the utility of C.OXT-G was whether drug-resistant viruses would emerge during the treatment. In this report, we established a C. OXT-G resistant strain, assessed its sensitivity and TK activity, and then studied the molecular basis for the reduction of the TK activity, using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and PCR direct sequencing technique. It may provide valuable information of the mode of drug action. It may also provide a basis for the evaluation of development of drug resistant strains in a clinical setting and help clinicians to face medical problems that might arise as a consequence of drug resistance with a deeper understanding of the molecular basis for this resistance.

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MATERIALS AND METHODS

Compounds, cells, and virus C.OXT-G was synthesised by Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Japan. VERO cells (African green monkey kidney cells) and HSV-1 were donated by the Department of Ophthalmology of Tokushima University, Japan.

Establishment of C.OXT-G resistant HSV-1 *in vitro* A suspension of VERO cells (5×10^5 cells) was dispensed into 60-mm dishes to obtain confluent monolayers and then they were inoculated with 100-200 PFU/dish of the PH strain of HSV-1. After absorption at 37 ºC for 1 h in 5 % CO_2 the inocula were removed and Eagle's minimum essential medium (EMEM, Sigma) containing 1 % fetal bovine serum (FBS) and different concentration (10.0, 1.0, and 0.1 μmol/L) of C.OXT-G was added. Further incubation carried out at 37 ºC in 5 % CO_2 for 2-3 d and CPE was examined under an inverted microscope. The infected VERO cells were frozen at -80 ºC for at least half an hour and then thawed to release the intracellular virus. Then, they were removed from the culture dishes and centrifuged. The supernatant was collected and inoculated on fresh VERO cell monolayers and incubated in the presence of the drug. The same process was repeated until the 10th passage.

Biological cloning Plaque purification was performed in order to obtain genetic homogeneity. The C.OXT-G^r strain (passage 10) was inoculated onto VERO monolayers at high dilution to obtain a few plaques. Well-isolated plaques were picked at random with Pasteur pipettes and were transported into aliquats containing 0.5 mL EMEM for stocking. At least 2 rounds of cloning were needed before next analysis.

Plaque-reduction assay The sensitivity of PH strain and C.OXT-G resistant strains (C.OXT-G^r) was determined by plaque-reduction assay in VERO cells. Two strains were inoculated into 60-mm dishes (100-200 PFU/dish). After absorption for 1 h, the free virus was removed and the infected cells were overlaid with 5 mL EMEM containing 1 % FBS, 1 % methylcellulose, and C.OXT-G in various concentration. After 3-d incubation, the cells were fixed with formalin, stained with crystal violet, and then plaques were counted. The dose of the drug that reduced the number of plaques to 50 % of the control (the 50 % effective dose, ED_{50}) was estimated from the plot.

Assay of TK activity Enzymatic assay of thymidine kinase activity was performed by the method of Summers *et al*[9] with a slight modification. Monolayers of LM cells (TK^-) were inoculated with the wild PH strain at 10 PFU/cell, the C.OXT-G^r strain and its clones. After incubation for 20 h, the cells were washed once with phosphate-buffered saline and then suspended in 2 mL of 0.02 mol/L Tris-HCl (pH 7.8) containing 1 mmol/L mercaptoethanol and 0.05 mmol/L thymidine, frozen and thawed, and then disrupted by ultrasonic vibration for 24 s. The sonicated cells were centrifuged at 8000×*g* (4 ºC) for 20 min, and the supernatant was used as a source of enzyme. The supernatant 40 μL was added to 120 μL of a solution containing 0.15 mol/L Tris-HCl (pH 7.8), 16 mmol/L $MgCl_2$, 16 mmol/L ATP, 25 mmol/L NaF, 8 mmol/L creatine phosphate, 1 kU/L creatine kinase and 0.2 mmol/L [^{3}H]thymidine (1.85×10^{11} Bq/mol; ICN Biochemicals). The reaction mixture was incubated for 30, 60, 90, and 120 min, and then 20 μL aliquots were applied to 2 cm×2 cm squares of DEAE paper (Whatman DE81). The papers were dried and their radioactivity was measured with a liquid scintillation counter (LSC-602, Aloka, Japan).

DNA samples preparation Culture supernatant 10 μL of PH strain and C.OXT-G^r strain were mixed with 90 μL of proteinase K solution (0.25 g/L proteinase K, 0.25 % SDS, 5 mmol/L edetic acid, and 10 mmol/L Tris-HCl, pH 8.0). After being incubated at 56 ºC for 2 h to digest the protein and at 95 ºC for 10 min to inactivate proteinase K, the mixture was diluted 10 times with ultra pure water and used as samples for PCR-SSCP analysis.

PCR-SSCP analysis According to our previous studies[10,11], seven pairs of primers were used for PCR, each of which was designed to amplify a part of TK gene of HSV-1 (Fig 1). Sample DNA 1 μL was amplified with 10 pmol of each primers, 0.25 nmol each of dNTPs, and 0.25 units of *Taq* DNA polymerase (Takara, Ohtsu, Japan) in the buffer supplied by Takara. Following an initial denaturation of sample DNA at 95 ºC for 1 min, 35 cycles each consisting of denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, and extention at 72 ºC for 1 min were carried out in a thermal cycle (GeneAmp PCR System 2400, Perkin-Elmer, Norwalk, CT). Resultant PCR product 5 μL was mixed with 4 μL of formamide dye mixture (95 % formamide, 0.05 % xylene cyanol and 0.05 % bromophenol blue) and 1 μL of 10 mmol/L edetic acid in 0.5 mol/L NaOH, heated at 95 ºC for 3 min and thereafter directly placed on ice to prevent reannealing of the single strand product. The samples (3 μL/lane) were then applied to the ready-to-

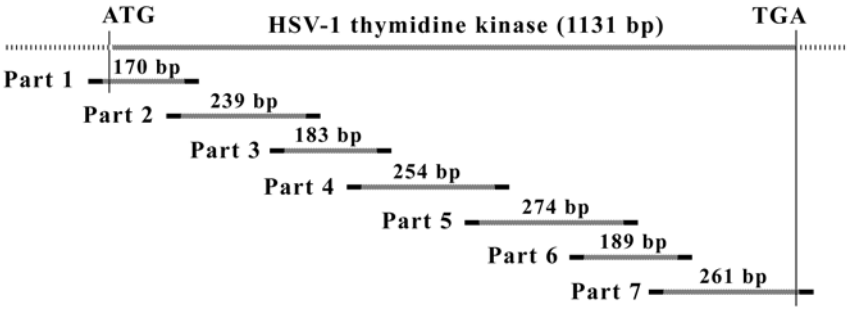

Fig 1. Schematic presentation of the location of DNAs amplified by PCR. Bold lines on both sides of each DNA indicate the pairs of primers. Sizes of amplified DNAs are shown in base pairs.

use mini gel supplied by genegel excel 12.5/24 kit (Pharmacia Biotech) and electrophoresis was performed on GenePhor eletrophoresis unit (Pharmacia Biotech) with ready-to-use buffer strips at 600 V, 25 mA for 120 min at 15 ºC. The gel was silver stained with the help of Hoefer automated gel stainer and PlusOne DNA silver staining kit (Pharmacia Biotech).

For DNA polymerase PCR-SSCP, the primers were designed to amplify the seven overlapping fragments of DNA polymerase coding region based on sequences reported previously[12,13] (Tab 1 and Fig 2). The conditions of PCR-SSCP were the same as that of TK gene.

DNA direct sequencing DNA direct sequencing was carried out using a pair of primers which were designed to amplify part 3 and 4 of the TK gene. One primer had the M13 forward consensus sequence at

Tab 1. Primers used for PCR-SSCP analysis on DNA polymerase of HSV-1.

Part	Primer	Sequence	Size
1	Pu[1]pol-1	5'-AATCCCACCTGAACGAGCTG-3'	115
	Pd[2]pol-1	5'-GGGCCGTACTGTTTCACAAG-3'	
2	Pupol-2	5'-CGACATGTACGGGATTATAACC-3'	287
	Pdpol-2	5'-TCTGCTGGCCGTCGTAGATG-3'	
3	Pupol-3	5'-ACCTGAGCTATCGCGACATC-3'	309
	Pdpol-3	5'-GCTGCGGCCGGACGCTTG-3'	
4	Pupol-4	5'-GGGGAGGACGAGGACGAAC-3'	242
	Pdpol-4	5'-GCCTCCAGGTGCGCCACT-3'	
5	Pupol-5	5'-CTGTTCTTCGTCAAGGCTCAC-3'	222
	Pdpol-5	5'-CGCGGCAACGTGCAGGCA-3'	
6	Pupol-6	5'-GGCCTTCGAACAGCTCCTG-3'	168
	Pdpol-6	5'-ATGTGGCTCGCCATCTTGTC-3'	
7	Pdpol-7	5'-TGCGAAAAGACGTTCACCAAG-3'	140
	Pdpol-7	5'-GCCCTGGAGGTGCGGTTGA-3'	

1 and 2: an upstream and a downstream, respectively.

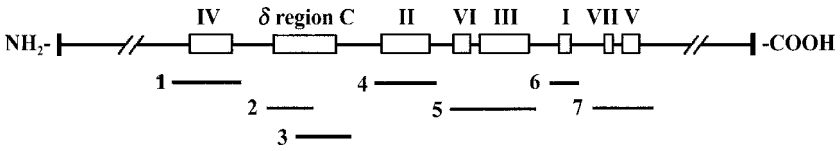

Fig 2. A schematic diagram of the HSV-1 polymerase region showing the location of the amplified fragments in relation to the conserved region in an approximated amino acid scale.

the 5' end and the other with the M13 reverse consensus sequence placed at the 5' end (Tab 2). PCRs were performed under the same conditions as for PCR-SSCP analysis as described above except for the total volume of 50 μL. The amplified fragments were gel-purified and the DNA was extracted from the gel. After sequencing reaction with a dye primer cycle sequencing FS ready reaction kit (Perkin-Elmer), nucleotide sequences were determined by an automated DNA sequencer model 377 (Perkin-Elmer).

RESULTS

Sensitivity of PH and C.OXT-G^r strains to C.OXT-G PH strain, C.OXT-G^r strain and its clones were assayed for plaque reduction by serial dilutions of C.OXT-G (Tab 3). The ED_{50} for the resistant strain and its clones were 12.92, 18.22, 6.86, and 30.27-fold (average 17.08-fold) greater than that for the wild strain.

TK assay The thymidine kinase activity in infected cells and uninfected cells was measured. In the wild PH strain, viral TK activity increased markedly, but in C.OXT-G resistant strain and its clones, reduced (Fig 3 and Tab 3).

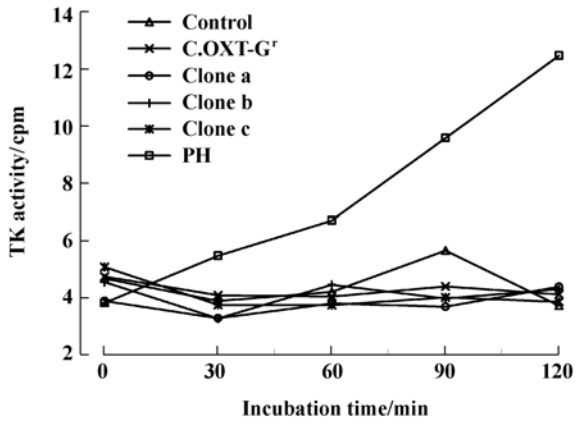

Fig 3. TK activity of the supernatants of LM (TK$^-$) cells infected with the PH strain, C.OXT-G^r strain and its clones.

Tab 3. Sensitivities to C.OXT-G and TK activities of the viruses.

Virus	ED_{50}[1)]/mol·L^{-1}	TK activity[2)]/cpm
PH strain	1.85×10^{-7}	12.200×10^{-3}
C.OXT-G^r	2.39×10^{-6}	3.855×10^{-3}
C.OXT-G^r (clone a)	3.77×10^{-6}	4.119×10^{-3}
C.OXT-G^r (clone b)	1.27×10^{-6}	4.032×10^{-3}
C.OXT-G^r (clone c)	5.60×10^{-6}	3.594×10^{-3}
Control (LM cell)		3.465×10^{-3}

1) C.OXT-G dosage required to inhibit plaque formation by 50 % of control.
2) TK activities of the viruses after incubation at 37 ºC for 120 min.

PCR-SSCP analysis and DNA sequencing PCR-SSCP analysis of the wild PH strain, the C.OXT-G^r strain and its clones showed altered migration patterns in the gel in part 3 and part 4 (Fig 4) and the migration patterns of the three clones were the same as that of the resistant strain. It indicates that the mutation may be at the area covered by both part 3 and part 4 of TK gene (between 415 and 437), or one mutation in part 3 while another in part 4 (Fig 1). This was proved by the DNA direct sequencing with the primers listed in Tab 2, which revealed a nucleotide deletion of G at position of 430 (Fig 6). This deletion caused a frameshift and generated a TGA stop codon at position 182 of the TK protein. PCR-SSCP analysis on DNA polymerase gene showed no difference among the PH strain, the C.OXT-G^r strain and its clones.

DISCUSSION

Since C.OXT-G is an antiviral drug that is already used for the treatment of herpetic keratitis and other HSV-mediated diseases[4,5,14], it is clearly important to

Tab 2. Primers used for PCR direct sequencing analysis.

Primer	Sequence
DSPu3[1]	5'-TGTAAAACGAGGCCAAGTGGGGGCTTCCGAGACAATC-3
DSPd4[2]	5'-CAGGAAACAGCTATGACCCAGGCGGTCGAGTGTCTGT-3'

[1] Upstream and [2]downstream primers for direct sequencing analysis of part 3 and 4 of the TK gene.

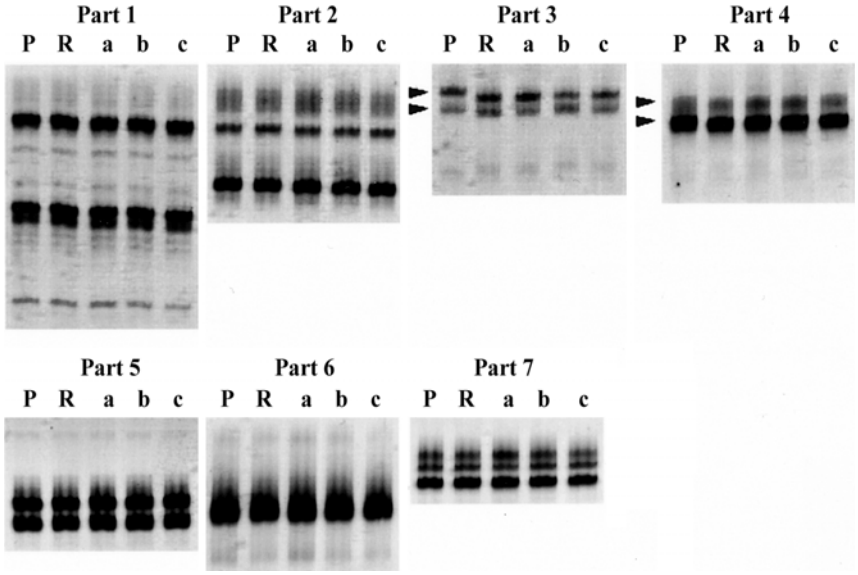

Fig 4. PCR-SSCP analysis of the TK gene of the PH strain, C.OXT-G^r strain and its clones. In comparison with the wild PH strain (lane P), mobility-shifted bands of C.OXT-G^r (lane R) and its 3 clones (lane a, lane b, lane c) can be detected in part 3 and part 4 of the TK gene.

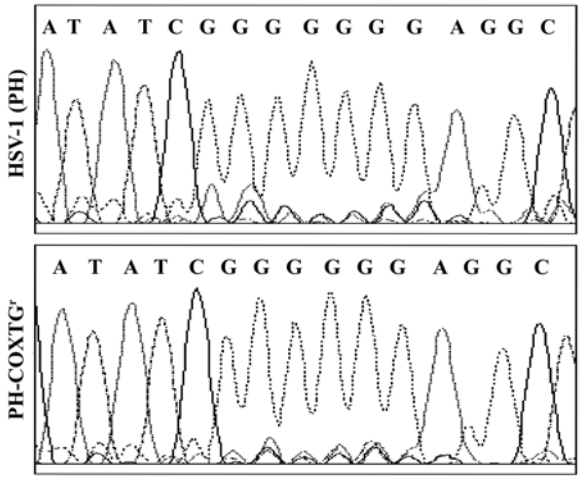

Fig 5. Fluorescence-base DNA sequencing of part 3 and part 4 of the TK gene. A deletion of G at position 430 caused a frame shift in C.OXT-G^r strains.

characterize resistant virus variants that may arise under the influence of the drug. Studies on the mode of action of the drug have pointed out the activity of C.OXT-G against HSV at least partially depends on phosphorylation by the virus-induced TK[15], and it suggests loss or alteration of TK activity could result in resistance. In our study, serial passage of sensitive wild PH strain of HSV-1 in C.OXT-G 10 μmol/L results emergence of drug resistant virus. The development of resistance correlates with diminished TK expression. The result constitutes strong evidence that mutation in the TK gene of the virus confer resistance to C.OXT-G.

In order to elucidate the precise nature of the mutation, the PCR-SSCP analysis combined with PCR-direct sequencing technique was applied. In comparison with the sequence of the wild PH strain, C.OXT-G^r strains had a single mutation consisting a deletion of G at position 430 of the TK gene. This subtle change, followed by a frameshift and a premature termination, was responsible for the above TK$^-$ phenotypein C.OXT-G^r strains. The possibility of the mutation due to a PCR artifact should be low, since it was found in all the 3 different clones that were sequenced. We found that the drugresistance to C.OXT-G may appear due to the deficiency of TK activity caused by a single mutation in the TK gene of HSV-1. The conclusion was in keeping with the previous reports on studies of resistant viruses to ACV[11], BVDU[16], and DHPD[10,17].

However, in some rare cases TK mutation can be detected together with DNA polymerase mutation while the latter contributed to the drug resistance. A HSV mutant containing a TK mutation and a standard TK enzyme assay on extracts of cells infected with this mutant gave the result that the mutant was TK-negative. The standard interpretation would be that the mutant was drug-resistant due to a TK defect. In fact, the mutant's TK defect was little if it had any effect on its drug sensitivity. Instead, the mutant was a recombinant virus containing a DNA polymerase mutation that conferred the drug resistance[18]. In order to exclude

this situation, we performed PCR-SSCP analysis on DNA polymerase. It showed no difference between the wild PH stain of HSV-1 and the C.OXT-G^r strains. Thus, TK of HSV-1 was the only target for C.OXT-G and contributed to its selectivity.

Since the coding region of the TK gene is 1131 bp, detecting point mutation by conventional sequencing methods is time consuming, expensive, and laborious. We applied a simplified, rapid, and non-radioactive PCR-SSCP analysis by which migration bands with abnormal mobility could be easily detected in less than 6 h. By cutting out the gel containing the DNA of positive part of the TK gene, the mutated sequences could be determined quickly.

It is anticipated that the study of C.OXT-G resistant mutations in the laboratory will aid clinicians in evaluating problems due to the potential development of drug resistant virus strains. Because of the frequent polymorphisms of the TK gene[11], an altered migration pattern on SSCP analysis of clinical samples does not always mean altered TK activity or drug-resistant mutation. The present method can be used to accumulate data on polymorphisms in TK gene of clinical isolates so as to help identifying resistant TK mutations not only to C.OXT-G, but also to other antivirus drugs. Furthermore, the present method should be useful when control parental strains are available for comparison, for example during serial observation of persistent or recurrent HSV infection seen in immunocompromised hosts.

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