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Original article

Prevention of the interaction between HVEM, herpes virus entry mediator, and gD, HSV envelope protein, by a Keggin polyoxotungstate, PM-19

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Abstract

One of the Keggin-type heteropolyoxotungstates ($K_7[PTi_2W_{10}O_{40}]6H_2O$:PM-19) is a potent inhibitor of the replication of herpes simplex virus (HSV) both standard strain 169 and the thymidine kinase-defective strain YS-4C-1 in vitro and in vivo. HSV envelope protein, gD, is necessary for virus entry into the cells. Some cellular molecules, such as HVEM, were reported to act as cofactors during the viral entry step. We determined whether PM-19 prevents these interactions between HSV-gD and HVEM. These activities were investigated using the Ciphergen and BIACORE system. Using a protein chip, many kinds of gD-specific binding proteins were captured, but these proteins could not be identified. Several proteins in these gD-binding proteins were inhibited its interaction with gD due to the presence of PM-19. Using the BIACORE system, the affinity of PM-19 to gD was low, because PM-19 has no direct inactivation activity against the virion. The specific binding of HVEM to the gD was shown as KD of 1.1e-9. The affinity of PM-19 for HVEM was high (KD:2e-9). To determine the competitive binding, the PM-19 (10 μg/ml) and several concentrations of HVEM solution mixtures were injected over the gD-fixed sensor surface. Each binding signal was stable in the range of approximately 270–300 RU. In the case of the addition of PM-19 to HVEM solution, the binding signals were elevated by PM-19 dose dependently. These results suggest that the bindings of PM-19 to gD are not disturbed by the presence of HVEM. PM-19 prevents the interaction between HVEM and gD.

Keywords: BIACORE; Herpes simplex virus; Polyoxometalate

1. Introduction

Almost all of the antiviral agents are nucleotide derivatives and easily induce drug-resistant variants from a parent virus [1]. We determined how a non-nucleotide derivative of an anti-HSV agent possesses a new action mechanism.

We have already found out that one of the Keggin-type heteropolyoxotungstates ($K_7[PTi_2W_{10}O_{40}]6H_2O$:PM-19) is a potent inhibitor of the replication of herpes simplex virus (HSV), both standard strain 169 and the thymidine kinase-defective strain YS-4C-1, at a non-toxic concentration in vitro [2].

As for the anti-HSV activity of PM-19, we have already demonstrated the following.

1. The 50% plaque-inhibiting concentration (EC_{50}) for HSV-2 strains for both 169 and YS-4C-1 were 4.2 μg/ml each [3].

2. PM-19 inhibited the viral penetration of cells and the second round of infection [2].

3. PM-19 had no activity in terms of the direct inactivation of HSV virions [4].

4. Pre-treatment of cells with PM-19 prior to infection enhanced the antiviral potency almost 10-fold compared with treatment of the cells only after infection, according to a quantitative PCR method [4,5].

To find the optimal application of PM-19 for virus infection in vivo, we investigated the therapeutic effect of PM-19 on skin lesions induced by infection with HSV-2, the acyclovir resistant strain, YS-4C-1, in mice and guinea pigs, respectively. PM-19 also inhibited the skin lesions induced by YS-4C-1 of HSV in vivo [6].

In a recent paper, it was demonstrated that the HSV envelope protein, gD, is necessary for virus entry into the cells. Also, some cellular molecules, HVEM (TNF receptor super family), nectin1,2 and 3-O-sulfated heparin sulfate were reported to act as cofactors at the step of viral entry [7–11].

In the present study, we determined whether PM-19 prevents these interactions between HSV-gD and cell surface

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membrane proteins, HVEM, as ligands. These inhibitory activities of interaction were investigated using the biological protein chip, Ciphergen, and surface plasmon resonance technology, the BIACORE system. PM-19 prevents the interaction between HSV-gD and HVEM at the cell surface.

2. Materials and methods

2.1. Cells and virus

Vero cells were used throughout the experiments. The cells were cultivated in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co. Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum. Chinese hamster ovary (CHO) cells were used as an HSV-resistant and HVEM-nonexpressed cell line. HSV type2, strain YS-4C-1 was kindly given to us by Dr. Mori, and it is a clone of a clinical source showing resistance to acyclovir and is deficient in thymidine kinase [12].

2.2. HSV envelope protein, gD and cell surface membrane protein, HVEM

HSV-1 glycosylated ectodomain of gD (319 a.a.) antigen was purchased from CORTEX Biochem Inc. (CA, USA). Recombinant human HVEM was purchased from TECHNE Corp. (MN, USA).

2.3. Antiviral compounds

Anti-HSV active and non-active polyoxometalates, PM-19 and PM-8, for use as reference compounds, were synthesized by Dr. Yamase et al. [13]. Acyclovir (Zobirax) was obtained from the pharmacy of Keio University Hospital.

2.4. Detection of the interactive proteins into cell surface membrane against HSV-gD using a protein chip, Ciphergen

HSV envelope protein, gD, was fixed on a spot of protein chip that was already coated with gD-Ab. Cell surface membrane proteins were prepared from the cultured Vero cells with a Proteo Prep Universal Extraction kit (SIGMA-Aldrich Japan, Tokyo, Japan). Vero cells-derived cell surface membrane proteins were incubated with the gD-fixed spot for 1 h on the protein chip. PM-19 was added to this reaction. After PBS was used to wash out the un-coupled proteins, gD-associated proteins were captured on a chip spot and analyzed using SELDI-TOF-MS.

2.5. Analysis of the interaction between HSV-gD and HVEM using surface plasmon resonance technology, the BIACORE system

The binding affinity and kinetics of gD and HVEM were measured using surface plasmon resonance with a BIACORE 2000 (Biacore, Uppsala, Sweden) instrument containing a CM5 sensor chip. HSV-gD or HVEM were immobilized onto each of the flow cells, using NHS/EDC amine coupling chemistry. Flow cells were activated for 7 min with a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.1 M 3-(N.N-dimethylamino)-propyl-N-ethylcarbodiimide at a flow rate of 20 μl/min. HVEM and gD as ligands were injected over each of the flow cells for 2–3 min, which resulted in a surface density of 1000–1500 resonance units (RU). Surfaces were blocked with a 7-min injection of 1 M ethanolamine–HCl at pH 8.5. The running buffer used for kinetic experiments contained 10 mM HEPES at pH 7, and 150 mM NaCl (HBS-N) purchased from BIACORE. All experiments were performed at 25° C at a flow rate of 20 μl/min. Each analysis cycle consisted of (i) the serial dilution of each analysis being injected for 2 min over both flow cells, the Logan-immobilized and the reference cells, (ii) a 3 min stabilization period, (iii) the regeneration of the Logan surface with a 30 s injection of 30 mM NaOH, (iv) a 3 min stabilization period. The signal was monitored as the ligand-immobilized flow cell minus the reference cell. The data from the buffer blank was subtracted from all of the sample run. The normalized data were then simultaneously fit to a 1:1 binding with mass transport model using steady state affinity analysis in BIA evaluation 3.1 software to obtain Kass and Kdiss parameters.

3. Results

3.1. Prevention of the interaction between gD and the cell surface membrane proteins by PM-19 (protein chip analysis, Ciphergen)

The interaction between HSV-gD that was fixed by the antibody to a spot on the Ciphergen chip and the crude proteins of the cell surface membrane were measured using SELDI-TOF-MS. The HSV-gD on the spot captured many proteins of the cell surface membrane on Vero cells, as shown in Fig. 1 (black line). PM-19 was added to this reaction system at a final con-

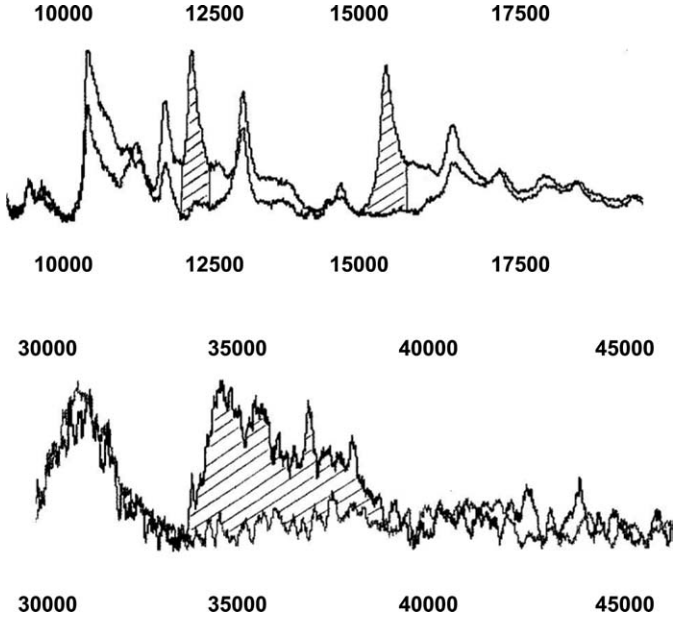

Fig. 1. Prevention of the interaction between gD to cell surface membrane protein by PM-19 using the Ciphergen protein chip system. Vero cells-derived cell surface membrane proteins incubated with the gD-fixed spot for 1 h on that protein chip. PM-19 was added to this reaction. After PBS was used to wash out the un-coupled proteins, gD-associated proteins were captured on a chip spot and were analyzed using SELDI-TOF-MS.

centration of 10 μg/ml and prevented the interaction between gD and several specific binding proteins, almost all 12,500, 15,300 and 34,000–38,000 MW proteins (Fig. 1; as shown in shaded line). In the case of using CHO cells, these gD-specific binding proteins were not cleared (data not shown).

3.2. Binding kinetics and affinity of HVEM or PM-19 to gD, gD or PM-19 to HVEM (BIACORE system analysis)

Using the BIACORE system, the binding kinetics and affinity of gD to analytes and analytes to HVEM were measured and shown in Table 1. The affinity of HVEM to gD was higher by about two-orders than the KD of PM-19. The specific binding of gD or acyclovir to the fixed HVEM could not be detected. PM-19 and PM-8, a compound non-active towards HSV, showed higher (KD: 2e-9) and lower (KD: 8e-7) affinity to HVEM, respectively (Table 1 and Fig. 2a). As shown in Fig. 2b, it was clear that the specific binding of PM-19 to HVEM disappeared due to the addition of 3 mM EDTA and were not influenced by the running buffer that contained 1.5 mM Ca^{2+}. However, the presence of Ca^{2+} ion induced the delay of the dissociation of PM-19 to HVEM (Fig. 2).

Table 1
Binding affinity of PM-19 to gD or HVEM measured by BIACOREa

Ligands	Analytes	KD (M)
gD	HVEM	1.12E-9
	PM-19	4.07E-07
HVEM	gD	No response
	PM-19	2.00E-09
	PM-8	8.00E-07
	Acyclovir	No response

3.3. Competitive binding of HVEM or PM-19 to gD (BIACORE system analysis)

The competitive binding of HVEM and PM-19 to the immobilized-gD were determined using the BIACORE system. In the case of the immobilized-HVEM, the specific binding of gD was not detected. Therefore, to each competitor solution (PM-19 at 10 μg/ml, or HVEM at 100 ng/ml) was added several concentrations of HVEM or PM-19, and each mixed solutions was injected over the gD-fixed sensor surface. The binding signals of PM-19 and HVEM observed after injections were approximately 220 and 100 RU, respectively, and the residual

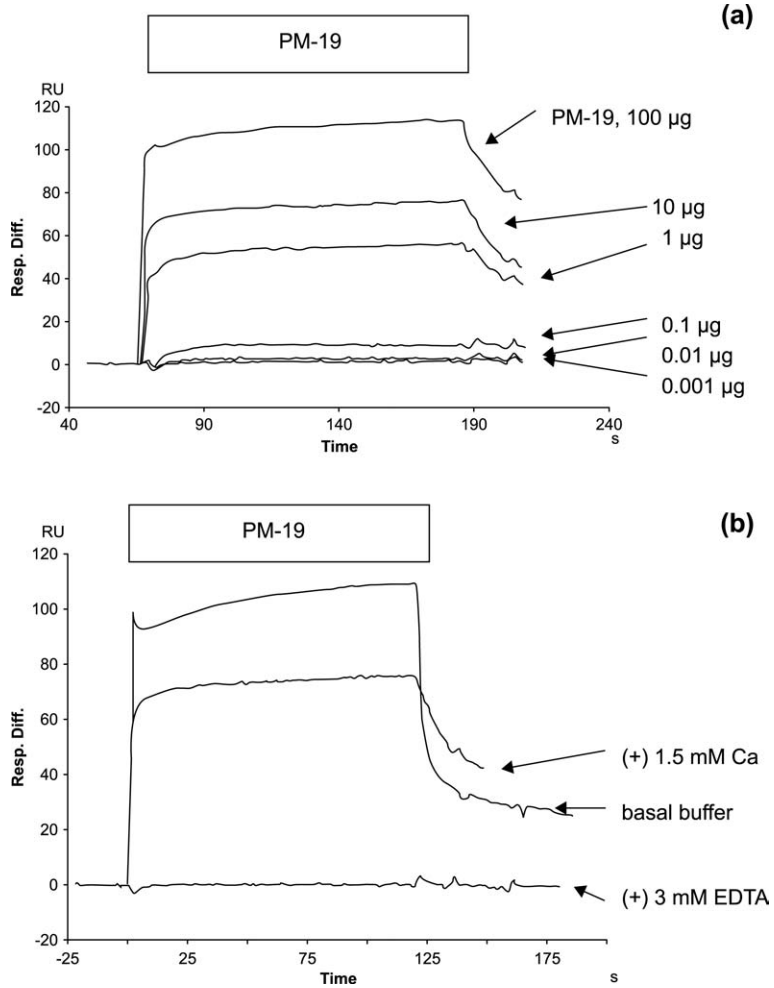

Fig. 2. Analytical biosensor analysis of the binding of PM-19 to HVEM. (a) The binding kinetics of PM-19 to HVEM. (b) The influence of 1.5 mM Ca^{2+} or 3 mM EDTA-addition into the running buffer to the binding of PM-19 to HVEM.

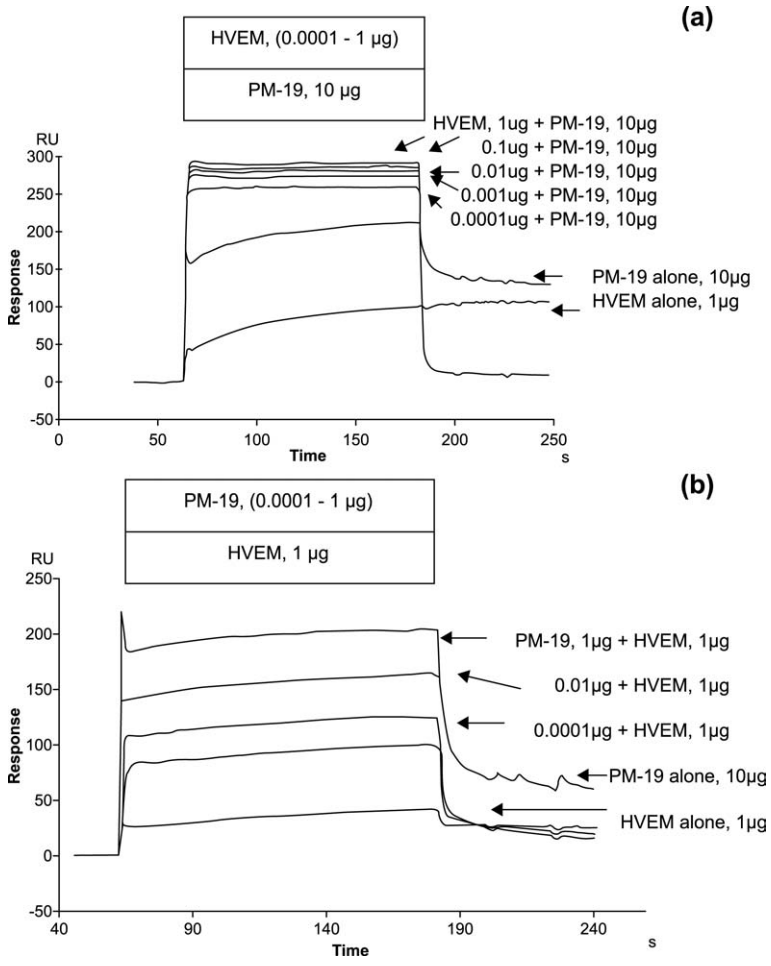

Fig. 3. The competitive binding of PM-19 and HVEM against the immobilized-gD. (a) The binding signals of the PM-19 (10 μg/ml) solutions to which were added several concentrations of HVEM (b) The binding signal of the HVEM (100 ng/ml) solutions to which were added several concentrations of PM-19.

binding signal of HVEM was not changed after 3-min dissociation. The binding signals of the PM-19 solutions to which were added several concentrations of HVEM were stable, and approximately 270–300 RU (Fig. 3a). In the case of the addition of PM-19 to HVEM solution, the binding signal was elevated depending on the dose of PM-19 (Fig. 3b).

4. Discussion and conclusion

PM-19 has been proven to be the most potent inhibitor of the replication of HSV at non-cytotoxic concentrations. The anti-HSV activity of PM-19 was given full play to its ability mainly at the virus entry stage. One of the HSV envelop proteins, gD, and several cellular cofactors are necessary at the stage of HSV entry into the cells [7–11].

In this paper, we determined that whether the specific binding protein to gD could be detected and whether PM-19 prevented the interaction between HSV-gD and HVEM as a main cofactor.

Using a protein chip, many kinds of gD-specific binding proteins were captured, but these proteins could not be identified. Several proteins among these gD-binding proteins were inhibited its interaction with gD due to the presence of PM-19 (Fig. 1). On analysis of the CHO cell membrane, gD-specific binding protein was not detected. These phenomena suggest that HSV-resistance depends on the expression of virus receptor.

As shown in Table 1, the affinity of PM-19 to gD was low, because PM-19 has no direct inactivation activity against the HSV virion. The specific binding of HVEM to the immobilized-gD was shown as a KD of 1.1e-9. However, the specific binding of gD to the immobilized-HVEM was not recognized. It was suggested that the conformation of the immobilized-HVEM was changed and the binding sites of gD or PM-19 against HVEM were different, individually. The affinities of PM-19 and PM-8 to HVEM were both high and low. It may be suggested that the antiviral activities of PM compounds were caused by the affinity to cell surface receptors. On the other hand, the low affinity of acyclovir to HVEM may be caused by an action mechanism as a terminator of DNA synthesis. The binding of PM-19 to HVEM disappeared due to the chelate compound and did not need the Ca^{2+} cation. It seems that PM-19 simply binds to ligand with an ionic bond. However, a residual binding signal of PM-19 to HVEM was delayed by the presence of the Ca^{2+} ion (Fig. 2). These phenomena suggest that the Ca^{2+} ion influences the dissociation of

PM-19 to HVEM, and then Ca^{2+} ions are always present under physiological conditions. We have already showed that the pre-treatment of cells with PM-19 prior to infection enhanced the antiviral potency almost 10-fold compared with treatment of the cells only after infection [13]. The pre-treatment of the cell with PM-19 may remain bound to a cell surface cofactor, such as HVEM.

To determine the competitive binding, the PM-19 (10 μg/ml) and several concentrations of HVEM solution mixtures were injected over the gD-fixed sensor surface. Each binding signal was stable in the range of approximately 270–300 RU. And in the case of the addition of PM-19 to HVEM solution, the binding signals were elevated dose dependently with PM-19. These results suggest that the bindings of PM-19 to gD are not disturbed by the presence of HVEM. Otherwise, the binding site of PM-19 to gD may be different from the site of HVEM. However, PM-19 prevents the interaction between HVEM and gD.

In this study, a gD-specific binding protein could not be identified using the protein chip. However, these proteins should be collectable using the BIACORE system and molecular weights and amino acid sequences of these proteins will be possible to determine by mass and MS/MS analysis [14,15]. In the recent paper [16], it was demonstrated that the carbohydrate-binding molecules, defensin, inhibit viral fusion and entry. These approaches are currently in preparation.

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