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Research Article

Screening and Mechanism of Trapping Ligand Antagonist Peptide for Chemokine Receptor US28 of Human Cytomegalovirus

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Abstract

Purpose: *The aim of the present study was to develop peptide H9 as an efficient antagonist of human cytomegalovirus (HCMV) chemokine receptor US28.*
Methods: *US28 gene was amplified from HCMV, and a stable expression system was constructed using NIH/3T3 cells. Interaction between peptide H9 and receptor US28 was tested by enzyme-linked immunosorbent assay. Flow cytometry was used to determine intracellular concentrations of Ca^{2+}, and the possible role of H9 as an antagonist was evaluated by anti-viral experiments.*
Results: *H9 interacts with the US28 receptor and prevents an increase of Ca^{2+} resulting from an interaction of chemokine with its receptor. Anti-viral assays showed that H9 could inhibit cytopathic effects of HCMV. AD169 infection (EC_{50} = 0.46 ng/ml), and the production of pp65 antigen were strongly inhibited with an EC_{50} value of 0.34 ng/ml.*
Conclusion: *The results demonstrate that H9 is an antagonist of US28, suggesting a possible role as a treatment for HCMV.*

Keywords*: Human cytomegalovirus, US28, Peptide H9, Trapping receptor/ligand, Antagonist*

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INTRODUCTION

Human cytomegalovirus (HCMV) is a widespread human virus that can infect immuno-compromised patients (e.g., HIV-infected patients, organ transplant recipients and newborn babies). Approximately 50 – 80 % of adults in North America and Europe, and approximately 100 % of adults in Asia and Africa, test seropositive for HCMV [1, 2]. During the last 20 yrs, HCMV infection has increased with increase in the numbers of immunocompromised individuals [3]. To date, no treatment has been developed for HCMV infection.

HCMV has developed numerous strategies for evading the human immune system. Four homologs of host G-protein-coupled receptors, including US27, US28, UL33 and UL78, are expressed after HCMV infection [4]. Among the mimics, US28 is the best characterized HCMV-encoded G-protein-coupled receptor. US28 is thought to act as a CC chemokine (CK) sink, to weaken immune responses at the infection site. US28 can also regulate intercellular transfer of HCMV via binding membrane-associated CXCL1 (fractalkine). Additionally, US28-induced vascular smooth muscle cell (SMC) migration might play a key role in induction of vascular diseases [3]. Therefore, US28 is regarded as an attractive target for the development of anti-HCMV therapy.

US28 amino acid sequence is approximately 30% homologous to the mammalian α- and β-receptors of leukocytes [5]. Although chemokine binding by US28 is different from known mammalian β chemokine receptors [6], it nonetheless plays an important role in β chemokine binding and calcium signaling in HCMV infected cells [7].

To avoid synthesizing a large number of peptides, bioinformatic methods were used in a previous study to predict the N-terminal active site and transmembrane domain of US28 [8] and a target synthetic polypeptide H22 was selected corresponding to residues 14-35 near the N-terminal region of US28 (Phe-Asp-Tyr-Asp-Glu-Asp-Ala-Th-Pro-Cys-Val-Phe-Thr-Asp-Val-Leu-Asn-Gln-Ser-Lys-Pro-Val). H22 exhibited no chemotaxic effect and could block the binding between physiologically active chemokine and receptor. Using a random phage library including 25 chemokines, 30 positive clones were selected. An enzyme-linked immunosorbent assay (ELISA) involving competitive blocking and competitive inhibition was used to identify clone No.5, which simulated human macrophage inflammatory factor-1β (hMIP-1β) when combined with synthetic peptide H22.

The aim of the present study was to design a peptide containing 9 amino acids (H9) on the basis of the structure of H22, and further evaluate its potential as an efficient antagonist of US28.

EXPERIMENTAL

Screening, modification and synthesis of peptide H9

A lead peptide was obtained by the phage display technique, composed of seven amino acids (LNAHCAL). After analysis using ProtParam, the target sequence VLNAHCALH (H9) was chosen. The properties of H9 were as follows: molecular weight, 977.1; isoelectric point, 6.88; good stability under standard pH and stable expression *in vitro*. The purity was determined using high-performance liquid chromatography. The molecular weight of H9 was determined using mass spectrometry.

Construction of cell lines US28-NIH/3T3 and ORF74-NIH3T3

After infection of strain AD169 (HCMV) for 72 h, the host cell with virus liquid was boiled for 10 min, and centrifuged at 12,000 rpm for 5 min. The supernatant was used for the PCR reaction. Primers of US28 were: 5'-GCCAAGCTTATGACACCGACGACGA-3' for

the upstream primer, and 5'-TGCTCTAGATTACGGTATAATTTGTGAGA CG-3' for the downstream primer. After purification, PCR products were digested with Xba1 and Hind III restriction enzymes, and ligated into plasmid pcDNA3.1. Using calcium phosphate, the US28 gene was transferred into NIH/3T3 cells. Recombinant clones were screened using culture medium containing 400 µg/ml G-418 disulfide. Cell line ORF74-NIH/3T3 was constructed in a similar manner. DNA was extracted from cell line BCBL infected with HHV8. ORF74 gene was amplified with the following primers: 5'-GTGGGATCCGATTACCCTGTTGTTAGCAC A-3' for the upstream primer, and 5'-AGCGTCGACTTATGTCATTTCCTGTGGAG A-3' for the downstream primer. The expression plasmid was constructed with PCR product ORF74 and expression plasmid pCEFL, then transfected into NIH3T3 cells.

Binding assay of H9 and receptors

Determination of calcium concentration in transfected cells

The concentration of cells was adjusted to 2×10^6/ml. Cells were treated as follows: for the chemokine group, hMIP-1β (10 ng/ml) was used; for the inhibitor group, 100 ng/ml H9 was used; for the inhibitor plus chemokine group, both hMIP-1β (10 ng/ml) and H9 (100 ng/ml) were used. After treatment for 24 h, cells were washed twice with serum-free RPMI1640, and the density of cells was adjusted to 1×10^7/ml. The molecular probe Fluo-3/AM was added at the final concentration of 10 µM. After incubation in the dark for 30 min at 37°C in a 5% CO_2 incubator, cells were washed with ice cold phosphate buffered saline (PBS) once, and resuspended with 0.5 ml PBS. Samples were tested using FACSCalibur flow cytometer after filtration. All results were expressed as mean ± standard deviation (SD).

Cross-linking of polypeptide H9 with receptors ORF74 and US28

Cross-linking of H9 with receptors ORF74 and US28 was determined using an ELISA assay. H9 was diluted to a concentration of 100 ng/ml, and aliquots (100 µl) were added into each well of a 96-well microtiter plate. Bovine serum albumin (BSA) and 10 ng/ml hMIP-1β were used as negative and positive controls, respectively. All assays were done in triplicate. After incubation overnight at 4°C, unbound polypeptides were removed by washing. A PBST solution (200 µl) containing 2% BSA was added to each well. Following incubation at 37°C for 1 h, the plates were washed. The cell density of US28-NIH3T3/ORF74-NIH/3T3 cells were adjusted to approximately 1×10^6/ml, and 100 µl was inoculated into each well of the 96-well microtiter plate. The pp65 monoclonal antibody was diluted with BSA-PBST solution at 1:2000, and 50 µl was added to each well. Plates were washed after 1 h incubation at 37°C. Then, the HRP-IgG was diluted with BSA-PBST solution at 1:1000, and 100 µl was added to each well. Plates were washed after 1h incubation at 37°C. 3,3',5,5'-tetramethylbenzidine chromogenic substrate solution (100 µl) was added to each well, then incubated at 37°C for 10 min. Stop solution (50 µl) was added to each well to stop the reaction, and absorbance was read at 450 nm.

Anti-HCMV effect of H9

Preparation of strains

Human embryonic lung fibroblast (HELF) cells in logarithmic phase were adjusted to a concentration of 1×10^6/ml, and 5-10 µl HCMV AD169 virus solution was added to the culture. Cytopathicity of cells was observed daily using a microscope, and the presence of pp65 in the supernatant was monitored every 3 days. Cells were tested for virus antigen (at approximately 8 days), then

digested, centrifuged, and cryopreserved at -80°C.

Cytotoxicity detection of H9

Cell toxicity of H9 was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny ltetrazolium bromide) assay. HELF cells in logarithmic phase were collected. The concentration of the cell suspension was adjusted to 5×10^4/ml, and 200 µl was added to wells of a 96-well plate. After incubated at 37°C in 5% CO_2 for 24 h, different concentrations of H9 were added to triplicate wells. No treatment of the HELF cells was used as the control. After 24 h of further incubation, an MTT working solution was added to each well, followed by a 4 h incubation; thereafter, 100 µl dimethyl sulfoxide was added to each well and incubated for 10 min. The supernatant was discarded, and 100 µl dimethyl sulfoxide was added to each well and incubated for 10 min. The absorbance at 490 nm was measured for each well, and the survival rate of cells was calculated.

Determination of virus titer after infection

The HCMV virus suspension was diluted by a factor of 10x under sterile conditions using stock solution. HELF cells in logarithmic phase were collected after trypsin digestion, and a cell suspension was prepared using 10% fetal bovine serum in Dulbecco's modified Eagle medium. Cell suspension (100 µl) was inoculated into wells of a 96-well culture plate, followed by inoculation with a dilution series of the virus. Each dilution was done in six duplicate wells. The 96-well plate was incubated at 37 °C in 5 % CO_2 for 7-10 days. Wells exhibiting the cytopathic effect were recorded until there was no longer any further development. Infection titer was defined as 50% of cells infected (median tissue culture infective dose, TCID50). The cumulative virus infection titer was obtained by the Reed-Muench method [9].

Inhibition of HCMV- induced cell lesions by H9

Serial dilutions of H9 were prepared, and 100 µl was added in triplicate to wells of a 96-well plate. Negative controls included wells with cells but no virus, as well as a ganciclovir-positive control. After adding 80 µl suspension containing HELF cells at 4×10^5/ml, and 20 µl diluted HCMV supernatant, the final volume was 200 µl for each well. The 96-well plates were incubated at 37°C in 5% CO_2 for 5 days, and observed for infected cell lesions and counted for the number of syncytial cytopathic effects (CPEs). CPE inhibitory rate and EC_{50} (50% effective concentration) were calculated according to the Reed-Muench method. The EC_{50} value was determined when 50% of the infected cells were inhibited by recombinant protein.

HCMV replication inhibited by H9

HELF cells infected with HCMV for 2 h, were inoculated in triplicate into wells of a 96-well plate, then cultured at 37°C in 5% CO_2 for 3 days. The supernatant collected after centrifugation was lysed with Triton X-100 using a volume fraction of 0.005. To detect the inhibitory effect of the drug on HCMV replication, HCMV-pp65 antigen was determined by ELISA. The replication inhibition rate and EC_{50} were calculated.

Data analysis

The results are presented as mean ±standard deviation. The data were analyzed by SPSS software version 16. Significant differences ($p < 0.01$) between groups were determined using the unpaired Student's t-test.

RESULTS

Detection and synthesis of H9

The purity of the H9 peptide, determined using high-performance liquid chromato-graphy, was more than 98%. Mass

spectrometry was performed to verify the sequence of H9. The molecular weight of synthetic peptide H9 was calculated as 977.5, which is consistent with the theoretical value.

Expression of receptor ORF74 and US28

PCR analysis of the US28 gene demonstrated that digestion with Hind III and Xba1 produced several single bands successfully (Figure 1), which was necessary to the following transfected assay. Western blot analysis of US28-NIH/3T3 showed a very high intensity band of molecular mass 4.1kDa after stained 6h and a low intensity at prestained (Figure 2). The results indicated the purity of US28-NIH/3T3. Two pictures of the ORF74 gene and ORF74-NIH/3T3 have been omitted.

Binding of H9 with US28

H9 prevents the elevation of Ca^{2+} induced by hMIP-1β

Figure 3 shows representative results of Ca^{2+} determined by flow cytometry. The data listed in Table 1 are the mean ± standard deviation of three independent experiments. The results showed that 10 ng/ml hMIP-1β can significantly increase the Ca^{2+} ($p<0.01$). Pretreatment with H9 (100 ng/ml) can prevent the increase of Ca^{2+} induced by hMIP-1β.

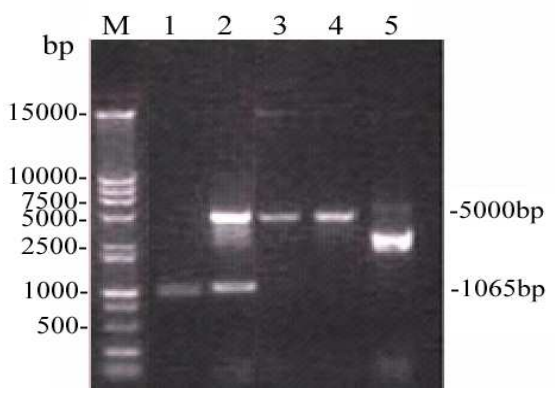

Figure 1: Digestion products of US28 and pcDNA3.1. M, molecular weight markers, 15000 and 2000 marker; 1, digestion products of US28 digested with Hind III and Xba1 after amplification; 2, double enzyme digestion products of pcDNA3.1-US28; 3, pcDNA3.1 digested with Hind III; 4, pcDNA3.1 digested with Xba1; 5, pcDNA3.1.

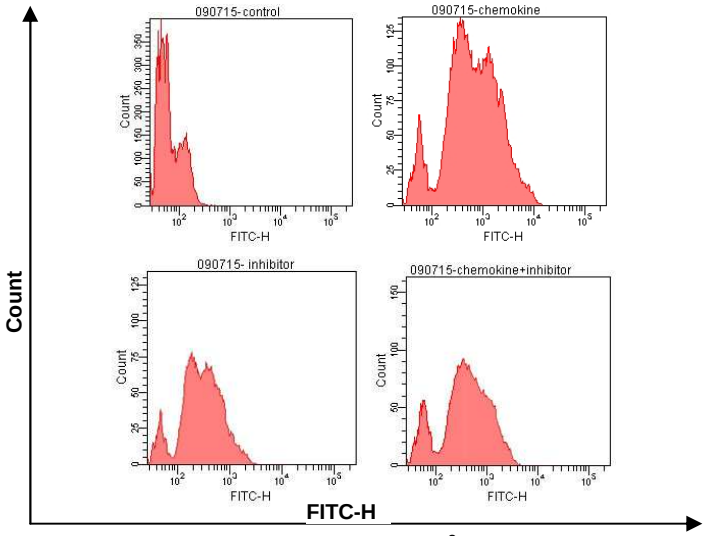

Figure 3: Flow cytometry of Ca^{2+} fluorescence

Flow cytometry was used for the detection of Ca^{2+} induced by hMIP-1β, H9 and H9+hMIP-1β respectively. The chemokine group (hMIP-1β) can significantly increase the Ca^{2+}, while the chemokine+inhibitor group (hMIP-1β+ H9) can down-regulate the concentration of Ca^{2+}.

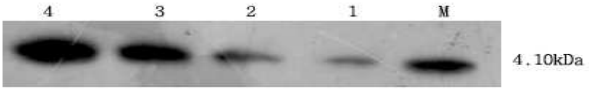

Figure 2: Western blot analysis of US28-NIH/3T3. M, pre-stained protein marker; 1, prestained protein; 2, poststained protein after 2 h staining; 3, poststained protein after 4 h staining; 4, poststained protein after 6 h staining.

Table 1: Effect of H9 on Ca^{2+}

Group	Fluorescence
Control	831±51
hMIP-1β (10ng/ml)	1453±77[a]
H9 (100ng/ml)	1074±30[a]
H9 (100ng/ml)+hMIP-1β (10ng/ml)	977±87[b]

[a] *p < 0.01 compared with negative control group;* [b] *p < 0.05 compared with positive control group,*

Identification of H9 and US28

The results on Table 2 show that the A_{450} value after H9 treatment is much higher than the value of the control after BSA treatment ($p<0.01$), indicating that a specific molecular interaction existed between H9 and US28/ORF74.

Anti-HCMV activity of H9

Cytotoxicity of H9

H9 did not exhibit significant cytotoxicity towards HELF cells, with survival rate of each treatment group more than 80%. However, the positive control involving ganciclovir treatment exhibited strong cytotoxicity towards HELF cells with an EC_{50} value of 1.86 μg/ml.

Virus titer

After 8 days of continuous culture, light microscopy showed lesions in cells, consistent with the replication of intracellular virus. No new changes were observed until 11 days after inoculation. At that time, $TCID_{50}$ was calculated when virus amplification was no longer increasing. $TCID_{50}$ was $10^{-2.48}$/100 μl for HCMV, respectively.

Table 2: ELISA assay of combinations between H9 and US28 or ORF74

Group	Concentration (ng/ml)	Mean ± SD (n=3)	
		US28	ORF74
hMIP-1β	10	0.593±0.085*	0.589±0.072*
H9	100	0.574±0.023*	0.580±0.041*
BSA	Control	0.108±0.016	0.112±0.014

** p < 0.01 compared with control ; SD = standard deviation*

H9 inhibition of HCMV-induced cytopathicity

Concentrations of peptide H9 (ranging from 0.08 - 250 ng/ml) were selected for the inhibition assay induced by HCMV. The results (not shown) indicate that H9 inhibited the formation of CPE in a dose-dependent manner. EC_{50} of H9 (0.46 ng/ml) was greater than that of the ganciclovir (GCV) group (0.68 ng/ml), i.e., positive control group.

H9 inhibits virus replication in HCMV-infected cells

H9 inhibited production of pp65 in HCMV-infected HELF cell culture supernatant in a dose-dependent manner. The amount of pp65 down-regulated by incubating the culture supernatants correlated with increase in the concentration of H9 or GCV (0.08∼250 ng/ml). The EC_{50} for H9 was 0.34 ng/ml, which was lower than the EC_{50} for the positive control (0.71 ng/ml).

DISCUSSION

The chemokine receptor is an attractive target for drug development, so researchers are making great efforts to study antagonists of this receptor. To date, there have been a variety of chemokine receptor antagonists entering phases of animal experiments or clinical studies [10-12]. Screening of antagonists for their ability to bind to virus-encoded chemokine receptors is an important

consideration when developing broad spectrum anti-viral therapies.

Recently, various small molecules with a similar structure to chemokines have been used as antagonists to inhibit inflammatory responses. These small molecules bind to the chemokine receptor, but do not initiate cytoplasmic signal transduction cascades. Normal physiological functions of chemokine receptors can be suppressed by these small molecule compounds. The N-terminal region of a chemokine is an important candidate site for development of antagonists, since it initially combines with receptors to start a series of signal cascades [13]. Crump *et al.* developed an SDF-1 analog by modifying Lys-1 and/or Pro-2 in the N-terminal region of SDF-1, using it as a HIV-1 receptor antagonist. [13].

Chemokine receptor US28 constitutes a class of receptors, which are similar to multiple herpes virus family members such as U12, U51 of HHV-7 and HHV-6, and ORF74 of HHV-8. Because US28 has the ability to bind various chemokines such as CC and CXC, this study characterized the binding of US28 to a broad spectrum of human chemokines. On one hand, the US28 receptor can bind to a variety of human chemokines. On the other hand, a library of broad spectrum chemokine peptides can be developed to bind to the CC and CXC chemokine classes. In the present study, significant differences in absorbance were observed between the H9-treatment group and negative control BSA-treatment group, suggesting a possible interaction between "trapping ligand" polypeptide H9 with the US28 receptor. Furthermore, studies of the effect of H9 on intracellular Ca^{2+} concentration show that pretreatment with H9 could efficiently prevent the elevation of Ca^{2+} concentration induced by hMIP-1β, which further demonstrated that H9 was an effective antagonist of US28. This result is supported by previous findings which showed that CC chemokines could increase the intracellular concentration of Ca^{2+} [14]. Given that pp65 is highly conserved in various strains of HCMV, and detection of pp65 has been considered as a standard method for monitoring the infection of HCMV [15], the inhibitory potential of H9 towards pp65 formation was evaluated to estimate the effect of H9 on HCMV replication. Our results showed that the "trapping ligand" H9 has good anti-viral activity, and inhibits replication of HCMV in a dose-dependent manner. Because US28 is similar to the chemokine receptor of other multiple herpes virus family members, H9 might also exhibit antagonistic effect towards these viruses.

An expression system for chemokine receptor US28 was successfully constructed *in vitro*. The binding of peptide H9 and US28 was confirmed, suggesting the signal blocking role of H9. In addition, H9 inhibited replication of HCMV *in vivo*. All these results indicate that peptide H9 could be a possible therapeutic antagonist for HCMV.

CONCLUSION

We characterized the peptide H9, which is an efficient antagonist of HCMV chemokine receptor US28. At the same time, the anti-viral test showed that H9 has the potential to inhibit the infection of HCMV. These results demonstrate that H9 is an antagonist of US28, and a potential candidate for treatment of HCMV.

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