日本財団補助金による
２０００年度日中医学学術交流促進事業

③．中国人研究者・医療技術者招聘助成事業

(3) ＨＩＶの細胞侵入機構の解明とその阻止

日本財団助成金による

2000 年度日中医学学術交流促進事業報告書

－中国人研究者・医療技術者招聘助成－

財団法人　日　中　医　学　協　会

理事長　中　島　章　　殿

年　　月　　日

研究発表中または研究中の本人のスナップ写真、及び発表論文等のコピーを添付

1．招へい責任者　服部俊夫　㊞

所属機関　東北大学大学院感染病態学分野　職名　教授

所在地　〒980-8574 仙台市青葉区星陵町1-1　電話　022-717-8220

招へい研究者氏名　凌　虹

所属機関　中国ハルピン医科大学微生物学教室　職名　助教授

研究テーマ　HIVの細胞侵入機構の解明とその阻止

2．日本滞在日程

平成12年4月10日より　8月末の期間

研究室に於て研究をした。

3．研究報告

別紙書式に従い、報告本文4000字以上(英文は2600語以上)で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

論文発表に当っては、*日中医学協会助成事業－日本財団助成金による*旨を明記して下さい。

HIV の細胞侵入機構の解明とその阻止

来日研究者　凌虹　(Hong Ling)
中国ハルピン医科大学　微生物学教室・助教授
招聘者　服部俊夫・東北大学大学院　感染病態学分野・教授

要旨

エイズの原因ウイルスである、HIV-1 の感染機構を解析する為に外膜糖蛋白である gp120 の V3 loop 由来ペプチドの HIV-1 感染に及ぼす影響を観察した。異なる HIV-1 株、BH10 (X4 strain) ADA (R5 strain) および 89.6 (X4R5 strain) 由来の V3 loop を作成した。またビオチン化した BH10 由来 V3 ペプチドも作成した。感染系として、ルシフェラーゼレポーター遺伝子と HXB2, ADA 及び 89.6 の どれかの外膜蛋白を用いた偽ウイルスを U87/ CD4/ CXCR4 あるいは U87/ CD4/ CCR5 に感染させて用いた。. V3-BH10 は HXB2 に対し感染促進作用を示し、たが V3-ADA と V3-89.6 はそれらの作用を示さなかった。同様に検索すると若干のオーバーラップがあるものの、それぞれの合成ペプチドは由来ウイルス特異的に感染促進作用を示した。.Virus Gprotein (VSV-G) をコートした偽ウイルスにはそのような促進作用は認められなかった。ビオチン化した BH10 由来 V3 ペプチドは T 細胞株、末梢血リンパ球、マクロファージ、等の様々な細胞に強く結合した。またこの結合は非ビオチン化 V 3 ループを添加することにより促進した。これらのことは V3 loop がウイルス感染に対して positive に作用することを明らかにした、最初の報告である。

Key words: V3-loop, HIV-1, peptide

Name: Hong Ling

Institution in China: Associate professor, Department of Microbiology, Harbin Medical University

Abstract

We investigated if V3 loop of envelope gp120 of HIV-1 can interfere the HIV-1 entry into the corresponding target cells. V3 loop of different HIV-1 strains including X4 strain, BH10, R5 strain, ADA and X4R5 strain, 89.6 were made. As a control non-looped peptide, BH10/CA in which both cysteine residues were replaced by alanine was synthesized. The biotinylated V3 peptides for BH10 and BH10/CA were also synthesized. For entry assay, the pseudotyped HIV-1 HXB2, ADA and 89.6 bearing Luciferase reporter gene were constructed by cotransfection of pNL-LucE-R- and one of the corresponding envelope gene into 293T cells. Binding ability of the biotinylated V3 BH10 and CA to the CEM/CCR5 was tested using PE-streptavidin staining via FACS analysis. U87/CD4/CXCR4 or U87/CD4/CCR5 cells were used as the target cells for the entry assay and the luciferase activity was detected 2 days after incubation of the peptides and the pseudotyped viruses. T140, which is a derivative of T22, was used as a control peptide. The results showed that when the peptides were added to the cells simultaneously with pseudoviruses, V3-BH10 and V3-BH10/CA showed enhancement effect on HIV-1 HXB2 infection, and this effect has apparent dose-dependence. The maximum percentages of enhancement are 93.6% and 88.2%, respectively, while peptides V3-ADA and V3-89.6 did not show the same effect on HXB2 infection. However, the peptide T140 showed inhibitory effect. For HIV-1 ADA infection, both V3-ADA and V3-89.6 can significantly enhanced HIV-1 ADA infection. For HIV-1 89.6 infection, only V3-89.6 can significantly enhanced HIV-1 89.6 infection no matter the target cells bearing CCR5 or CXCR4 as the coreceptors. When the retroviral pseudotype bearing vesicular stamotitis Virus G protein (VSV-G) was used for observing the effect of the V3-peptides on virus entry, the viral infectivity was not affected by any of V3-peptides. Biotinylated V3-BH10 and V3-BH10/CA were found to bind to not only T cell line, PBMC and MDM, also human astroglia cell line U87, U87 transferred and stably expressed CD4 or coexpressed both CD4 and CXCR4 or CCR5 molecules on the cell surface, U87.CD4 and U87.CD4.CXCR4 or U87.CD4.CCR5. This demonstrated the binding does not depend on CD4 and coreceptor presence. While biotinylated V3-ADA binding ability is limited. It could bind to monocyte, MDM, and the U87 cell lines but not T cell line. The results were identical with that of the effect of V3-peptides on the coreceptors expression or direct competition. We did not see any increasing coreceptor expression and the peptides did not inhibit the binding of the antibodies anti-coreceptors used in present experiment

Key words: V3-loop, HIV-1, peptide

Research report

The effect of the peptides derived from the V3-loop of HIV-1 on the virus infection

Purpose

V3-loop is the neutralizing epitope and cell tropism determinant of HIV-1. To clarify its functions on viral entry the looped peptides derived from the V3-loop of X4 (BH10), R5 (ADA) and X4R5 (89.6) HIV-1 strains were designed and synthesized and their influences on HIV-1 entry into corresponding target cells were observed via a sensitive one round fusion system, replication defective retroviral vector system.

Methods

1. Peptides design and synthesis

The amino acid sequences of the peptides derived from the V3 loop of T-tropic (III BH10), M-tropic (ADA) and Dual-tropic (89.6) HIV1 strains are listed in table 1. All V3-peptides were synthesized using an automatic peptide synthesizer 430A. After synthesis, the protecting group was removed, and the peptides were cleaved from the supporting resin with trifluoromethanesulfonic acid. The obtained peptides were purified then by high-performance liquid chromatography (HPLC). A disulfide bond was made for V3-BH10, ADA and 89.6. The formation of disulfide bond was verified by the determination of triol groups by using a fluorescent probe, ammonium 7-fluobenzo-2-oxa-1,3-diazol-4-sulfonate and L-cysteine as a standard. The looped peptide with an intramolecular disulfide bond was disalted and purified by HPLC. The fractions containing the peptide were collected and lyophilized. The peptide purity was investigated and exceeded 95%. The rsult of Ion spray spectrum analysis of the all peptides indicated that these peptides had their theoretical molecular weights. As the control, a non-looped peptide V3-BH10/CA was synthesized by substituting both of the cysteins of V3-BH10 with two alanines. To determine if the V3 loop could bind to the target cell surface directly, the biotinylated V3-peptides including Bio-V3-BH10, BIO-BH10/CA and BIO-V3-ADA were designed and synthesized by linking biotin molecules to the N-termini of the original V3-peptides.

The peptide quantity was carried out using BIO-RAD DC protein assay kit.

Table 1 The sequence of V3-peptides

peptides	Sequence
V3-BH10	EINCTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHCNIS
V3-BH10/CA	---A----------A---
V3-89.6	ESVV----------RRLS-··------YARRN-IGDI--------
V3-ADA	----------H-··-------Y-T-E-IGDI-------

2. Pseudoviruses production and p24 antigen determination

The effects of the peptides on HIV-1 entry and replication ware examined using pseudotype viruses containing luciferase reporter gene. The envelope expression plasmids pSM-HXB2, pSM-ADA and pSP272-89.6 containing the env gene of X4 HIV-1 strain, HXB2, R5 strain, ADA and the X4R5 HIV strain 89.6, and a retroviral expression vector bearing *P. pyralis* luciferase reporter gene, pNL4-3-Luc-E$^-$R$^-$, which contains a frameshift mutation near the 5'- end of *env* in the *NdeI* site of NL43, were used for generation of luciferase reporter gene-containing pseudotyped viruses.

These pseudotyped viruses were obtained 48 h after co-transfection of luciferase reporter gene-containing vector, pNL43-Luc-E-R-, and one of the Env-expressing vectors (pSMHXB2, pSMADA, pSP89.6) into 293T cells upon the transfection manufacture (LIPOFECTAMINE PLUS PEAGENT, GIBCO.BRL).

After transfection, the cell culture supernatant was collected and the amount of p24 antigen was determined using antigen capture ELISA. The p24 antigen of pseudoviruses was above 100 ng/ml.

3. The effects of V3 peptides on HIV infection

Human astroglia cell lines expressing CD4 antigen and coreceptor CXCR4 (cell line U87.CD4.CXCR4) or CCR5 (cell line U87.CD4.CCR5) were used as the target cells for X4, R5 and X4R5 viruses, respectively. G418 (Geneticin Disulfate, Wako Pure Chem., Osaka) and puromycin (Sigma, St. Louis, MO) were added as selection reagents. The viability of the cells was estimated using the trypan blue dye exclusion method.

For infection assay, pseudotyped viruses were used to infect target cells seeded in 24-well-plate ($4x10^4$ cells/ well) one day before the experiments. The V3 peptides were added at serial concentrations simultaneously with the pseudotyped viruses. After incubating the cells at 37°C for 1hour, the cells were washed in Hank's and incubated for another 48 h at 37°C. The cells were collected by treating in 150 μl/ well of 1X luciferase cell Culture Lysis Reagent (Promega, Madison,WI), and the luciferase activity of each lysate was measured

by luminometer (Bio Orbit 1251 luminometer, Turku, Finland). The luciferase activity (RLU, relative light unit) in cells treated with pseudotyped viruses only was used as the negative control. The enhancement percentage of the compounds were calculate as (RLU of the cells treated by compounds- RLU of the cells treated by medium without compounds)/ RLU of the cells treated by medium without compounds x 100%. T140, a derivative of T22 (antagonist for CXCR4) was used as a control peptide.

4. Binding of Biotinylabeled V3-peptides to the cells and FACS analysis

Human T cell line SupT1, MolT4, and CEM.CCR5, a cell line transfected with coreceptor CCR5 gene, human PBMC, monocyte derived macrophage (MDM), U87, U87/CD4 (U87 cell transfected with human CD4 gene), U87/CD4/CXCR4 and U87/CD4/CCR5 were used for binding assay.

$5X10^5$ of cells were incubated in RPMI 1640 containing 0.5% BSA and 0.01% sodium azide (incubation solution) and corresponding biotinylated-V3 peptides at different concentrations for 40 min at 4 °C. After being washed with PBS-BSA-NaN3 (washing solution), the cells were stained by phycoerythrin conjugate streptavidin(PE-streptavidin, S-886, Molecular probes, Inc. Oregon, USA) in the same buffer for 30min at 4 °C. After removing the PE-streptavidin by washing twice, the cells were resuspended in 1% formalin buffered solution and analyzed using a Flow cytometer (FACScan, Becton Dickinson Immunocytometry System, USA). The negative control consisted of cells treated with the PE-conjugated streptavidin but not preincubated with the biotinylated V3-peptides. 10000 cells were counted and the result was represented as the mean of fluorescent intensity of stained cells by PE-streptavidin-biotin.

5. The effect of the V3-peptides on the binding of the antibodies anti-coreceptors

The monoclonal antibodies anti-CXCR4, 12G5, MAB170, 171, 172 and 173, and anti-CCR5, 2D7, 5C7, MAB181, 182 were used in the experiments of the coreceptors expression and direct competition assay. Both of them are provided via *NIH AIDS research and references and reagent program*. FITC conjugated Goat F(ab') 2 anti-mouse Ig"s was purchased from BIOSOURSE INTERNATIONAL CORP.(Tagoimmunologicals, TM, California).

$5X10^5$ of CEM.CCR5 cells were incubated in RPMI 1640 containing corresponding V3 peptides at the concentration of 10nM, 100nM, 1 μM and 10 μM for 0min and 1 hours at 37 °C. After being washed twice, the cells were resuspended in washing solution containing mouse MAb anti-coreceptors and were maintained for 30 min at 4 °C. Subsequently, the cells were washed and stained by FITC-conjugated goat anti-mouse- F(ab')2 for 30 min at 4 °C. The

collected cells were washed twice and analyzed using Flow cytometer (FACScan, Becton Dickinson Immunocytometry System, USA).

Results

The results showed that when the peptides were added to the cells simultaneously with pseudoviruses, V3-BH10 and V3-BH10/CA showed enhancement effect on HIV-1 HXB2 infection, and this effect has apparent dose-dependence. The maximum percentages of enhancement are 93.6% and 88.2%, respectively, while peptides V3-ADA and V3-89.6 did not show the same effect on HXB2 infection. However, the peptide T140 showed inhibitory effect. For HIV-1 ADA infection, both V3-ADA and V3-89.6 can significantly enhanced HIV-1 ADA infection. For HIV-1 89.6 infection, only V3-89.6 can significantly enhanced HIV-1 89.6 infection no matter the target cells bearing CCR5 or CXCR4 as the coreceptors. When the retroviral pseudotype bearing vesicular stamotitis Virus Gprotein (VSV-G) was used for observing the effect of the V3-peptides on virus entry, the viral infectivity was not affected by any of V3-peptides, meaning the enhancement effect of the V3-peptides was envelope dependent. The results of the experiments by pretreating the target cells with the V3-peptides followed by washing out the compounds and infecting them with HIV-1 pseudoviruses showed the enhancement effect in the same manner indicating that the peptides can interact with some molecules on the target cell surface and the enhancement effect was involved in this interaction.

As the fact that the biotinylated V3-BH10 can bind to the surface of some of T, B cell line and peripheral blood monocytes was investigated previously (1), we are wondering if the binding ability is specificly limited within lymphocytes and monocytes, and if this binding is related tightly to the enhancement effect of the V3-peptides. We synthesized biotinylated V3-BH10, BH10/CA and ADA. The binding ability of these peptides to wide range of cells was examined. As expected, biotinylated V3-BH10 and V3-BH10/CA were found to bind to not only T cell line, PBMC and MDM, also human astroglia cell line U87, U87 transferred and stably expressed CD4 or coexpressed both CD4 and CXCR4 or CCR5 molecules on the cell surface, U87.CD4 and U87.CD4.CXCR4 or U87.CD4.CCR5. This demonstrated the binding does not depend on CD4 and coreceptor presence. While biotinylated V3-ADA binding ability is limited. It could bind to monocyte, MDM, and the U87 cell lines but not T cell line. The results were identical with that of the effect of V3-peptides on the coreceptors expression or direct competition. We did not see any increasing coreceptor expression and the peptides did not inhibit the binding of the antibodies anti-coreceptors used in present experiment

Discussion

The early molecular events associated with human immunodeficiency virus type 1 (HIV-1) infection have not been clarified. The HIV-1 glycoproteins play an essential role in virus attachment and entry. Interaction of gp120 with the CD4 molecules on the target cells as the receptor is confirmed to be the early step of the entry. Chemokine receptors, mainly CXCR4 or CCR5, are involved in the subsequent step of virus-cell attachment as a coreceptor for HIV-1. Chemokine receptor binding is believed to trigger additional conformational changes in the viral envelope glycoproteins that ultimately lead to the fusion of viral and target cell membranes. The V3-loop is thought to be involved in this chemokine-gp120 interaction (2,3, 4, 5).

In present works, we have found the enhancing effects of synthesized V3-peptides derived from different tropic HIV-1strains on their original viruses infection. The enhancement is viral restricted except that of peptide derived from X4R5 strain 89.6. The biotinylated V3-BH10 binding range is as wide as to CD4 positive, coreceptor expressing, and the cells, which do not bear CD4 or any of the coreceptors. The finding showed that there are other ligand molecules on cell surface except if the coreceptors were the binding target of the V3-loop.

The mechanisms of the enhancement effect of the V3-peptides on virus infectivity are exploring now.

References

1. Murakami, T., S. Matsushita, Y. Maeda, K. Takatsuki, T. Uchiyama, and T. Hattori. 1993. Application of biotinylated V3 loop peptides of human immunodeficiency virus type 1 to flow cytometric analysis and affinity chromatographic techniques. Biochemica et Biophisica Acta 1181:155-162.
2. Lapham, C.K., J. Ouyang, B. Chandrasekhar, N. Y. Kguyen, D. S. Dimitrov, and H. Golding. 1996. Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. Science 274:602–605.
3. Trkola, A., T. Dragic, J. Arthos, J. M. Binley, W. C. Olson, G. P. Allaway, C. Cheng-Mayer, J. Robinson, P. J. Maddon, and J. P. Moore. 1996. CD4 dependent, antibody sensitive interactions between HIV-1 and its co-recep-torCCR-5. Nature 384:184–187.
4. Ugolini, S., M. Moulard, I. Mondor, N. Barois, D. Demandolx, J. Hoxie, A. Brelot, M. Alizon, J. Davoust, and Q. J. Sattentau. 1997. HIV-1 gp120 binding to CD41 cells induces an interaction between CD4, gp120 and the chemokine receptor CXCR4. J. Immunol. 159:3000–3008.
5. Wu, L., N. P. Geradr, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A. A. Cardoso, E. Desjardin, W. Newman, C. Gerard, and J. Sodroski. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. Nature 384:179-183