Effect of Human Saliva on HIV-1 Binding and/or Entry to Target Cells

Etsuko Kumagai[1], Nobuko Mori[1], and Shinji Harada[2]

Abstract

Introduction: Human saliva is known to possess anti-HIV-1 activity, but how saliva inhibits HIV-1 infection of target cells has not been fully clarified. To clarify the effects of saliva on the HIV-1 infection process, we examined the effect of whole saliva on plasma membrane, viral receptor/coreceptor and actin filaments of HIV-1 target cells and HIV-1 infectivity.

Methods: HIV-1_{IIIB} infected cells and cell damage were evaluated using X-Gal staining and trypan blue staining. The amount of HIV-1_{IIIB} was measured using HIV-1 p24 antigen ELISA. CD4 and CXCR4 were measured using laser scanning confocal microscope and FACScan flow cytometry. Actin filaments were observed using laser scanning confocal microscope. Whole unstimulated saliva was collected from four healthy volunteers.

Results: Unboiled saliva retained its strong viscosity even after filtration through a 0.45-μm filter, and HIV-1 infection of MAGI/CCR5 cells was inhibited by 81.6 ± 7.3 (SD) %, even after mixing of HIV-1 and unboiled saliva. This rate was almost the same as that after incubation with HIV-1 plus unboiled saliva for 1 hour at 37℃. In contrast, boiled saliva with decreased viscosity barely had an effect; there was no significant difference in the amount of viral binding by MAGI/CCR5 cells. The cells were barely damaged after incubation for 60 min with unboiled and boiled saliva. The expression levels of CD4 and CXCR4 and distribution of the actin filaments were also unaffected.

Conclusion: These results suggest that the viscosity of whole saliva does not inhibit HIV-1 infection by blocking HIV-1 binding (specific and nonspecific) to target cells, but rather by blocking HIV-1 entry into target cells.

Key words: whole saliva, viscosity, HIV-1, inhibition, CD4, CXCR4, actin filaments

I. Introduction

In the 21 years that have past since the first outbreak of immunodeficiency virus (HIV), about 40 million people worldwide are now infected with HIV[1]. About 70% of HIV infection is transmitted by sexual contact, with infection through blood and vertical contagion between mother and child each representing only about 1% of cases[1]. Oral HIV transmission rates are especially low and HIV is not easily transmitted by kissing or dental procedures[2][3]. A number of salivary HIV inhibitors and CD4-negative mucosal epithelial cells in the oral cavity contribute to these very low oral HIV transmis-

Departments of Health Science[1] and Medical Virology[2], Kumamoto University School of Medicine

sion rates[4)-9)]. Anti-HIV antibodies in the saliva of seropositive individuals are thought to neutralize the virus[4)]. Moreover, mucin[5)6)], salivary agglutinin (Gp340)[7)], and secretory leukocyte protease inhibitor (SLPI)[8)] in the saliva of seronegative individuals are thought to have anti-HIV activity.

Infection of human cells by HIV-1 is initiated by the infection of viral envelope gp120 and specific molecules on the target cell surface. CD4 has been known as an essential receptor for HIV-1 binding and entry. Most primary isolates (R5 strains) use CD4 together with the CCR5 coreceptor, while T-cell tropic strains (X4 strains) use CXCR4 coreceptor, dualtropic strains (R5X4 strains) use both CXCR4 and CCR5 coreceptor[10)11)].

Actin is essential for maintaining cell morphology and function[12)13)]. Many bacteria modulate the actin cytoskeleton of eukaryotic host cells through attachment or invasion[14)15)]. It has also been reported that actin plays an important role in the maturation and release of HIV[16)]; however, the role of cellular actin in the process of HIV binding and cell entry have yet to be clarified.

In this study, in order to clarify the effects of saliva on the process of HIV-1 infection (binding and/or entry), we examined the effect of whole saliva on the plasma membrane, CD4, CXCR4 and actin filaments of HIV-1 target cells and HIV-1 infectivity.

Ⅱ. Materials and methods

1. Saliva collection and treatment

Whole unstimulated saliva was collected from four healthy volunteers. Half the saliva was left untreated, while the remainder was incubated at 56 or 100℃ for 30 min. All saliva samples were then centrifuged for 30 min at 1,500 × g at 4℃ then passed through a 0.45 μm MillexTM filter (Millipore, Bedford).

2. Cells

MGGI/CCR5 cells were derived from HeLa-CD4/LTR-β-Gal cells transfected with pZeoSV-CCR5 using the calcium phosphate method as previously described[17)]. The cells were cultured in Dulbecco's minimal essential medium (DMEM) (ICN, Costa Mesa, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamin, 100 IU/ml hygromycin B, 0.05 mg/ml of zeomycin, 100 IU/penicillin and 0.1 mg/ml streptomycin. Molt4 cells were grown in suspension in RPMI 1640 medium (Gibco) supplemented with 100 IU/penicillin, 0.1 mg/ml streptomycin, and 10% heat inactivated FBS[18)].

3. Preparation of virus stocks

ⅢB strains which is one of X4 strains were propagated persistently in HIV-$1_{ⅢB}$ chronic infected Molt4 (Molt4/HIV-$1_{ⅢB}$) cells[19)]. Cell-free viruses were obtained by filtration of the cell supernatants through 0.45-μm filters. Viruses were aliquoted on receipt and stored at −80℃ until use. p24 antigen levels of the virus were 28 ng/ml.

4. Multinuclear activation of a galactosidase indicator (MAGI) assay

One day before HIV-1 infection, MAGI/CCR5 cells (15×10^4) were seeded in a 3.5-cm dish. HIV-$1_{ⅢB}$ strains were mixed with an equivalent amount of saliva or DMEM with 10% FBS then incubated for 4 hours at 37℃ and the changes during that time monitored.

Cells were infected with 1 ml of HIV-1 plus saliva or HIV-1 plus medium for 1 hour at 37℃. Unbound viral particles and saliva were removed and the cells then cultured for 2 days at 37℃. After removing the supernatant, fixation and staining of HIV-1 infected cells were carried out using a previously described MAGI assay[6)20)].

5. Viral antigen assay

MAGI/CCR5 cells (15×10^4) were seeded in a 3.5-cm dish then cultured for one day at 37℃. After infection for 1 hour at 37℃ with 1 ml of HIV-1 plus unboiled or boiled saliva, cells were washed 3 times with DMEM then lysed with 0.5 ml of 1 % triton-X. The amount of HIV-1 and the level of protein were measured using HIV-1 p24 antigen ELISA (Zepton Metric, NY) and a BCA protein assay kit (PIERCE, Rockford), respectively. HIV-1 binding amount by MAGI/CCR5 cells was expressed as the amount of HIV-1 p24 antigen amount per 100 μg of protein.

6. Measuring the number of damaged cells

Preincubation of MAGI/CCR5 cells were carried out as for the viral antigen assay. After incubating for 5 to 60 min at 37℃ with 1 ml of saliva or DMEM with 10% FBS, the cells were washed with PBS (pH 7.2). The cells were stained with 0.2% trypan blue dye for 5 min then washed with PBS. Molt4 cells (20×10^4) were also incubated with unboiled saliva, boiled saliva or RPMI 1640 with 10% FBS for 5 to 60 min at 37℃ then stained with the trypan blue dye. The rate of damage of both cells was then deduced after counting both stained and unstained cells under a microscope.

7. Immunofluorescent staining of viral receptor and coreceptor

MAGI/CCR5 cells were seeded on a microscope cover glass (13-mm in diameter; Fisher, Pittsburgh, PA) in a 3.5-cm dish at 60×10^4 cells per dish. The following day, the cells were incubated with 1 ml of HIV-1 plus unboiled saliva or HIV-1 plus medium for 1 hour at 37℃. After removing unbound viral particles and saliva, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were incubated for 30 min on ice with anti-CXCR4 (12G5) antibody (R&D Systems), followed by 30 min incubation with a secondary rhodamine-labeled anti-rat IgG (Chemicom International, Temecula, CA). After washing, the cells were incubated for 30 min on ice with anti-CD4 antibody (Ortho Diagnostics, Raritin, NY), followed by 30 min incubation with a secondary FITC-labeled anti-mouse immunoglobulins (Capptl), and then washed with PBS. They were next mounted with immunofluorescence mounting solution (Molecular probes, Eugene, OR) and imaged using a laser scanning confocal microscope (Olympus FV500, Japan) equipped with a PlanApo 40× WLSM objective and Ar488 nm/He-Ne543 nm laser.

8. Flow cytometric analysis for viral receptor and coreceptor expression level

Preincubation and incubation with HIV-1 plus unboiled saliva of MAGI/CCR5 cells were carried out as for the viral antigen assay. Cells removed in 0.25% trypsin were incubated with anti-CD4 antibody, anti-CXCR4 antibody for 30 minutes on ice. After washing with PBS, they were then incubated with a secondary FITC-labeled anti-mouse

immunoglobulins for 30 minutes on ice. After fixing with 4% paraformaldehyde, the cells were analyzed using FACScan (Becton Dickinson).

9. Immunofluorescent staining of cellular actin

Preincubation, incubation with HIV-1 plus unboiled saliva and fixation of MAGI/CCR5 cells were carried out as for the immunofluorescent staining of viral receptor and coreceptor. Fixed cells were incubated with phalloidin TRITC-labeled mixed isomers from amanita phalloides (Sigma) for 1 hour at room temperature then washed with PBS. They imaged using the laser scanning confocal microscope equipped with a PlanApo 60× WLSM objective.

10. Measuring levels of Na

Levels of Na in the saliva were measured using a flame photometric detector (FLAME-30C; Nihon Bunko Co., Japan).

Ⅲ. Results

1. Cell damage by saliva

Both MAGI/CCR5 and Molt4 cell damages were measured with whole saliva from four healthy individuals. As shown in Fig 1 (A), the damage rate of MAGI/CCR5 cells barely increased, even when saliva treatment was carried out for 1 hour at 37℃. Unboiled saliva and boiled saliva damaged about 0.9 ± 0.5 and 1.1 ± 0.3% of cells after 1 hour, respectively. In contrast, when Molt4 cells were incubated for 5 min with unboiled or boiled saliva, damage rates were 78.9 ± 14.5 and 91.1 ± 7.0%, respectively, revealing that Molt4 cells were damaged more by boiled saliva than unboiled saliva (Fig. 1, B). In our previous report, we reported that unboiled whole saliva with Na levels of less than 20 mEq/l rapidly damaged Molt4/HIV-1_{IIIB} cells[6]. The Na levels of the saliva used here were less than 11 mEq/l.

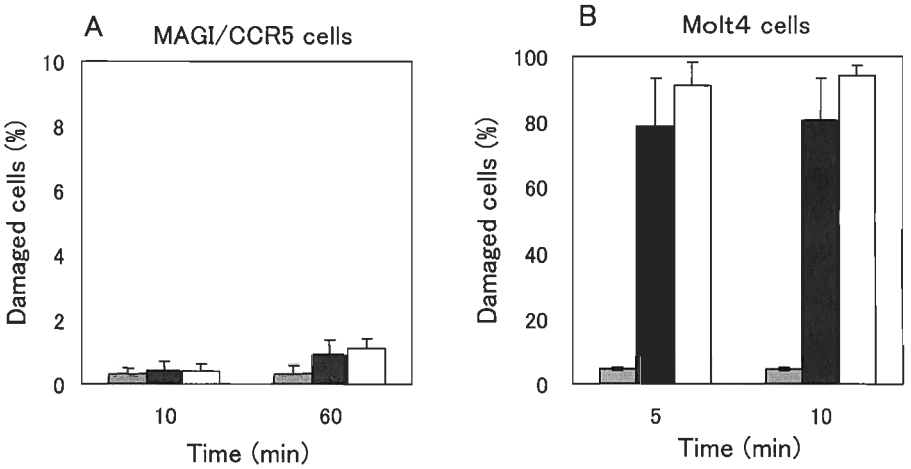

Fig. 1 Damage rates of MAGI/CCR5 cells (A) and Molt4 cells (B) in response to whole saliva. Both cell types were incubated with unboiled saliva(■), boiled saliva(□) or medium(□) at 37℃. The data are expressed as mean ± standard deviation (n=4).

2. Change in HIV-1 infection rate with the duration of HIV-1 plus saliva incubation

Change in the viral infection rate within the duration of HIV-1 plus unboiled saliva incubation was monitored for 4 hours. The HIV-1 infection rates on MAGI/CCR5 cells of HIV-1 incubated with medium or four unboiled salivas are shown in Fig. 2 (A). The HIV-1 infection rate of the HIV-1 plus medium was unchanged up until 4 hours at about 13%. On the other hand, that of the HIV-1 plus saliva group was very low, 1.2~3.4%, even after mixing of the HIV-1 and saliva. The HIV-1 infection rate tended to decrease with increasing duration of HIV-1 plus saliva incubation, and the HIV-1 infection rate after 4 hours incubation was 0.3~1.3%.

The rates of HIV-1 inhibition by four unboiled salivas are shown in Fig. 2 (B). Unboiled saliva inhibited HIV-1 infection of MAGI/CCR5 cells by 73.4~90.6%, even after mixing HIV-1 and saliva. The rate of HIV-1 inhibition after 4 hours incubation increased by 6.8~16.4% compared with that at 0 min.

3. Effect of saliva on HIV-1 infectivity and viral binding

The rates of HIV-1 inhibition and the amount of viral binding (specific and nonspecific) by MAGI/CCR5 cells after incubation with HIV-1 plus unboiled and boiled saliva are shown in Fig. 3. Unboiled saliva inhibited HIV-1 infection of MAGI/CCR5 cells by 81.6 ± 7.3%, but boiled saliva barely had an effect; however, there was no significant difference in the amount of viral binding by the cells. Using saliva warmed for 30 min at 56 ℃, the rate of HIV-1 inhibition was 73.0 ± 2.8%. Effect of this saliva on viral binding by the cells was not measured.

4. Effect of saliva on viral receptor and coreceptor

To examine whether inhibition of the HIV-1_{MB} infection was due to the decreased expression of viral receptors and coreceptors,

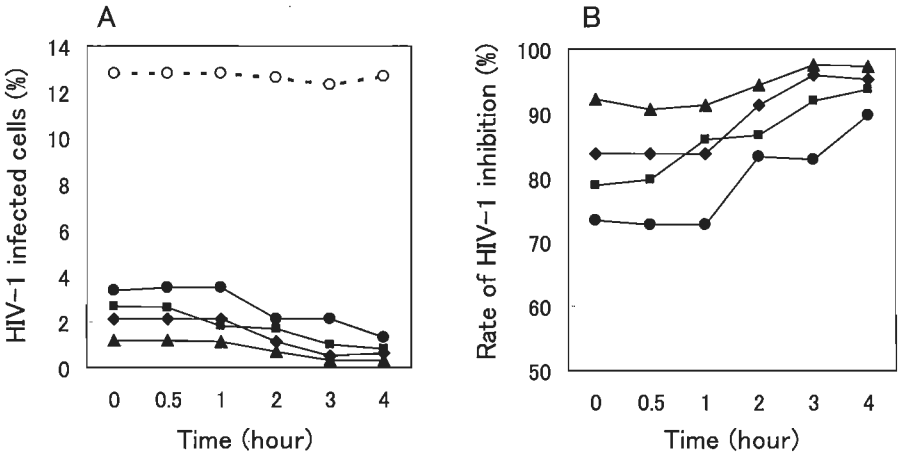

Fig. 2 Rates of HIV-1_{MB} infection (A) and inhibition (B) with duration of HIV-1 plus unboiled saliva incubation. A: HIV-1 was incubated with an equivalent amount of unboiled saliva (●, ■, ◆, and ▲) or medium (○) for 0.5 to 4 hour at 37℃. MAGI/CCR5 cells were incubated for 1 hour with these HIV-1. HIV-1 infected cells were counted 2 days postinfection. B: Data points were derived from each HIV-1 infection rate of HIV-1 plus unboiled saliva and HIV-1 plus medium in fig. 2 (A).

we examined effect of saliva on CD4 and CXCR4. The expression levels of CD4 and CXCR4 on MAGI/CCR5 cells were measured by FACScan flow cytometry. The mean expression levels ± SD of CD4 and CXCR4 on MAGI/CCR5 cells in the HIV-1 plus saliva group (n=4) were 84.4 ± 3.0 and 76.4 ± 3.5, respectively, while those of the HIV-1 plus medium group were 85.4 ± 1.6 and 76.7 ± 1.7, respectively. No significant difference was observed with regard to the expression levels of CD4 and CXCR4 between two groups.

The localizations of CD4 and CXCR4 on MAGI/CCR5 cells were observed by laser scanning confocal microscope. As shown in

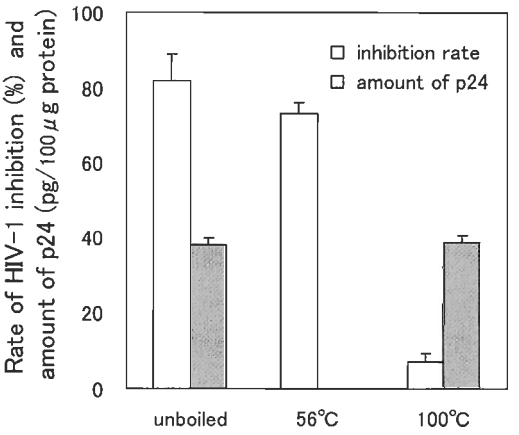

Fig. 3 Effect of whole saliva on HIV-1_{IIIB} infectivity and viral binding. MAGI/CCR5 cells were incubated for 1 hour at 37℃ with either HIV-1 plus unboiled saliva, HIV-1 plus saliva warmed at 56℃ for 30 min, or HIV-1 plus saliva boiled at 100℃ for 30 min. After culturing the cells for 2 days at 37℃, HIV-1 infected cells were counted. The amount of viral binding by the cells was examined using HIV-1 p24 antigen ELISA after incubation for 1 hour at 37℃ with HIV-1 plus unboiled or boiled saliva. The data are expressed as mean ± standard deviation (n=4).

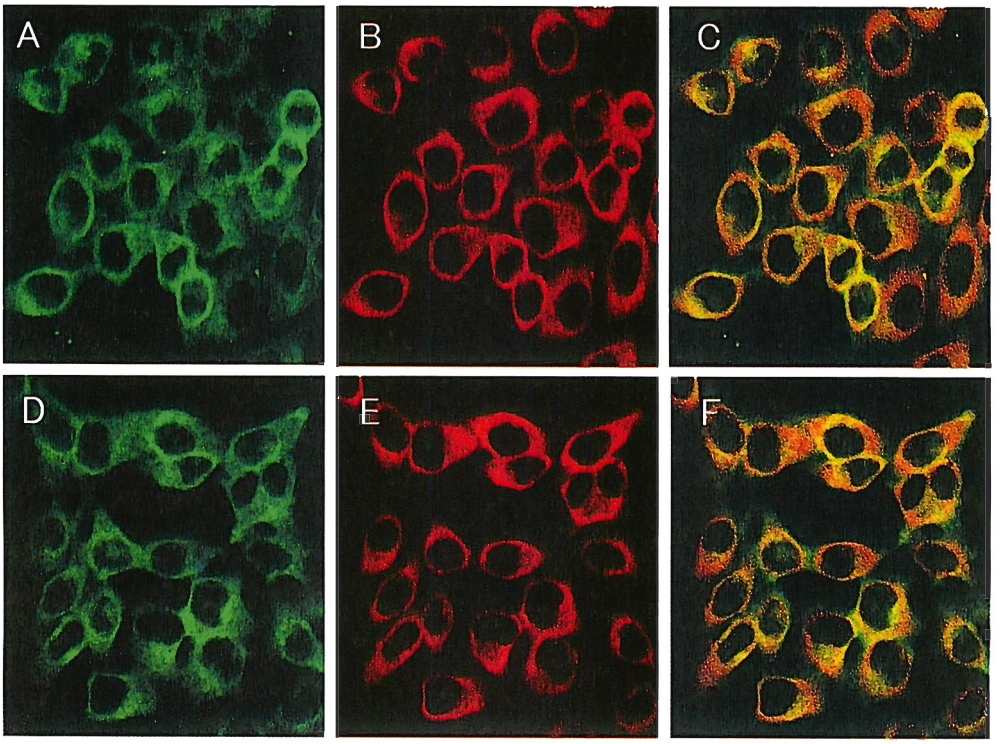

Fig. 4 Effect of whole saliva on localization of CD4 and CXCR4 on MAGI/CCR5 cells. Cells were incubated with HIV-1 plus medium (A, B, and C) or HIV-1 plus unboiled saliva (D, E, and F) for 1 hour at 37℃. Cells stained with anti-CD4 antibody plus secondary antibody conjugated with FITC (A and D, visualized as green) and anti-CXCR4 antibody plus secondary antibody conjugated with rhodamine (B and E, visualized as red). Colocalization of CD4 and CXCR4 is illustrated as yellow (C and F).

Fig. 4, CD4, CXCR4 and CD4/CXCR4 were homogeneously distributed on the cell surface (A-F, respectively). CD4/CXCR4-colocalized cells were over about 73% in the cells.

5. Effect of saliva on cellular actin

Effect of saliva on distribution of actin filaments was measured by laser scanning confocal microscope. As show in Fig. 5, the actin filaments of MAGI/CCR5 cells incubated with HIV-1 plus saliva extended forward toward the peripheral part of the cell, just like in cells incubated with the HIV-1 plus medium.

IV. Discussion

It has been reported that saliva causes cell damage by its lack of salt, saliva being just one seventh as salty as other bodily fluids[6)21)]. As mentioned, unboiled saliva with a Na content of less than 20 mEq/l rapidly damaged Molt4/HIV-1_{MB} cells[6)]. In this study, we examined the hypotonic action of whole saliva on HIV-1 target cells. Sensitivity to hypotonic action between adherent cells and unadherent cells was shown to be remarkably different. About 79% of unadherent Molt4 cells were damaged after incubation with unboiled saliva for 5 min. Boiled saliva with a reduced viscosity damaged the Molt4 cells more than unboiled saliva, damaging about 91% after 5 min. The difference in the hypotonic action on unadherent cells between these two saliva suggests that saliva viscosity might suppress the permeability of the plasma membrane. On the other hand, adherent cells such as MAGI/CCR5 cells were barely damaged even after incubation for 60 minutes with unboiled and boiled saliva, respectively.

Saliva-induced cell damage is weakened by isotonic fluids such as blood and mother's milk. Baron et al. reported that saliva diluted 25~75% with isotonic medium partially suppressed cell damage[21)]. We previously reported that the damage rate of Molt4/ HIV-1_{MB} cells was remarkably lowered when whole saliva was diluted by over 10% with PBS[6)].

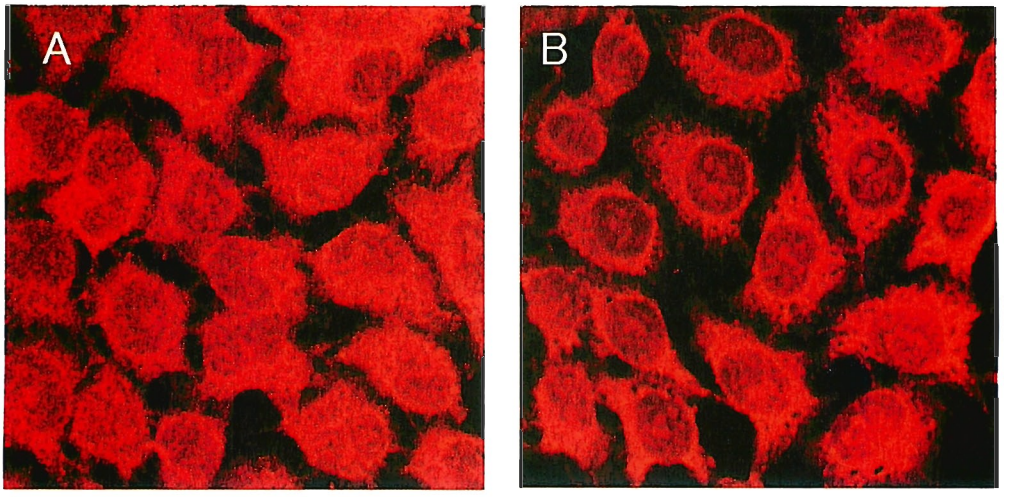

Fig. 5 Effect of whole saliva on distribution of actin filaments on MAGI/CCR5 cells. Cells were incubated with HIV-1 plus medium (A) or HIV-1 plus unboiled saliva (B). Actin filaments stained with phalloidin TRITC-labeled mixed isomers (visualized as red).

We think that saliva-induced inactivation of free HIV-1 may be also weakened by isotonic medium.

CD4 receptor and CXCR4 coreceptor are known to play important roles in binding and entry of X4 strains[10)11)]. It is also known that cellular actin plays an important role in maturation and release of HIV-1[16)]. Here, the expression levels of this viral receptor and coreceptor on MAGI/CCR5 cells and the distribution of actin filaments were not affected by unboiled saliva during the process of binding and entry of HIV-1 to target cells.

A number of salivary molecules that inhibit HIV infection have been identified[4)-8)]. Secretory leukocyte protease inhibitor (SLPI) appears to disrupt the HIV-1 infection processes soon after viral binding, but does not appear to act directly on the virus[8)]. Salivary mucin functions in the aggregation/entrapment of viral particles[5)6)], while salivary agglutinin (Gp340) mediates inhibitory activity by binding to HIV-1 gp120, involving a region different from the CD4-binding site on gp120[7)]. We previously showed how HIV-1 infection of MAGI/CCR5 cells was inhibited by 85～97% after filtration of HIV-1 plus unboiled saliva[6)]. In this study, unboiled whole saliva retained its strong viscosity even after filtration through a 0.45-μm filter, and HIV-1 infection of MAGI/CCR5 cells was inhibited by 81.6 ± 7.3 (SD) % even after mixing of HIV-1 and unboiled saliva. This rate was almost the same as that after incubation with HIV-1 plus unboiled saliva for 1 hour at 37℃. In contrast, boiled saliva with decreased viscosity did not block HIV-1 infection. Even when enzymatic activity was eliminated by warming for 30 min at 56℃, the saliva inhibited about 73.0 ± 2.8 (SD) % of the HIV-1 infection because viscosity was strong. We therefore hypothesized that unboiled saliva would inhibit viral binding (specific and nonspecific) by MAGI/CCR5 cells more so than boiled saliva; however, there was no difference in the amount of viral binding between cells infected with HIV-1 plus boiled saliva and HIV-1 plus unboiled saliva. These results suggest that the viscosity of whole saliva does not inhibit HIV-1 infection by blocking HIV-1 binding to target cells, but rather by blocking HIV-1 entry into target cells.

Whole saliva in humans is secreted at a rate of 1～1.5 L per day and 0.02～1.85 ml per min when not eating[22)]. *In vivo*, free HIV becomes wrapped in saliva and is then swallowed; therefore, the sojourn time of HIV in the oral cavity is very short, and thus oral HIV infection is prevented.

Collectively, we have shown that whole saliva does not inhibit HIV-1 infection by working on CD4, CXCR4 and actin of target cells, but rather by blocking HIV-1 entry into target cells.

Acknowledgements

We thank Dr. Y. Maeda for supplying MAGI/CCR5 cells, and Ms. M. Murakami, Ms. R. Osumi, Ms. S. Hayashi, and Ms. S. Mouri for technical support.

References

1) UNAIDS.: Report on the Global AIDS epidemic 2005, WHO 2005

2) Levy J, et al.: HIV in saliva. Lancet 2: 1248, 1988

3) Gooch B, et al.: Lack of evidence for patient-to-patient transmission of HIV in a dental practice. J Am Dent Assoc 124: 38-44, 1993
4) Mestecky J, et al.: Paucity of antigen-specific IgA responses in sera and external secretions of HIV-type 1-infected individuals. AIDS Res Hum Retroviruses 20: 972-988, 2004
5) Bergey, et al.: Interaction of HIV-1 and human salivary mucins. J Acqir Immune Defic Syndr 7: 995-1002, 1994
6) Kumagai E, et al.: Inhibitory effect of human saliva on HIV-1 infectivity. Jpn J Clin Immun 24: 152-159, 2001
7) Wu Z, et al.: Gp340 (SAG) binds to the V3 sequence of gp120 important for chemokine receptor interaction. AIDS Res Hum Retroviruses 20: 600-607, 2004
8) McNeely TB, et al.: Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription. Blood 90: 1141-1149, 1997
9) Baba TW, et al.: Infection and AIDS in adult macaques after nontraumatic oral exposure to cell-free SIV. Science 272: 1486-1489, 1996
10) Bazan HA, et al.: Patterns of CCR5, CXCR4 and CCR3 usage by envelope glycoproteins from human immunodeficiency virus type 1 primary isolates. J virol 72: 4485-4491, 1998
11) Berger EA, et al.: Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol 17: 657-700, 1999
12) Civelekoglu G, et al.: Modeling actin filament reorganization in endothelial cells subjected to cyclic stretch. Bull Math Biol 60: 1017-1037, 1998
13) Disatnik MH, et al.: The bi-directional translocation of MARCKS between membrane and cytosol regulates integrin-mediated muscle cell spreading. J Cell Sci 117: 4469-4479, 2004
14) Dramsi S, et al.: Intracellular pathogens and the actin cytoskeleton. Annu Rev Cell Dev Biol 14: 137-166, 1998
15) Yoshida S, et al.: Exploiting host microtubule dynamics: a new aspect of bacterial invasion. TRENDS Micro 11: 139-143, 2003
16) Perotti MA, et al.: Directional budding of Human immunodeficiency virus from monocytes. J virol 70: 5916-5921, 1996
17) Maeda Y, et al.: Involvement of both the V2 and V3 regions of the CCR5-tropic human immunodeficiency virus type 1 envelope in reduced sensitivity to macrophage inflammatory protein 1 alpha. J Virol 74: 1787-1793, 2000
18) Koyanagi Y, et al.: Estabilishment of a high production system for AIDS retroviruseswith a human T-leukemic cell line MOLT-4. Cancer Lett 30: 299-31, 1986
19) Harada S, Koyanagi K, Yamamoto N.: Infection of HTLV-Ⅲ/LAV in HTLV-1-carrying cells MT-2 and MT-4 and application in a plaque assay. Science 1985: 229: 3240-3246
20) Kimpton J, et al.: Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an . integrated β-galactosidase gene. J Virol 66: 2232-2239, 1992
21) Baron S, et al.: Why is HIV rarely transmitted by oral secretions? Arch Intern Med 159: 303-309, 1999
22) Becks H, et al.: Human saliva XⅢ. Rate of flow of resting saliva of healthy individuals. J Dent Res 22: 391-396, 1943