Antagonistic Effect and Mechanism of ADS-J1 on SEVI-Enhanced HIV-1 Transmitted/Founder Virus and Chronic Control Virus Infection Post-print

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Abstract

Objective: To investigate the enhancement of HIV-1 transmitted/founder (TF) and chronic control (CC) virus infection by semen-derived enhancer of virus infection (SEVI) and the mechanism by which ADS-J1 antagonizes SEVI-enhanced viral infection. Methods: Thioflavin T (ThT) assay was used to verify that PAP248-286 can self-assemble into SEVI amyloid fibrils. A pair of TF and CC infectious clone viruses were amplified. SEVI was mixed with TF and CC viruses respectively to infect TZM-bl cells, and luciferase activity was measured after 72 h to evaluate the fold enhancement of viral infection by SEVI. SEVI was treated with different concentrations of ADS-J1, then mixed with TF and CC viruses respectively to infect TZM-bl cells, and luciferase activity was measured after 72 h to investigate the antagonistic effect of ADS-J1 on SEVI-enhanced TF and CC virus infection. Subsequently, ADS-J1 was mixed with viruses to infect TZM-bl cells, and luciferase activity was measured after 72 h to verify the direct antiviral effect of ADS-J1. Finally, SEVI was treated with different concentrations of ADS-J1, and its Zeta potential was measured to preliminarily explore the mechanism by which ADS-J1 antagonizes SEVI-enhanced TF and CC virus infection. Results: ThT assay results demonstrated that PAP248-286 can self-assemble into SEVI amyloid fibrils. SEVI could significantly promote TF and CC virus infection ($P<0.05$). ADS-J1 not only significantly antagonized SEVI-enhanced TF and CC infection ($P<0.05$), but also directly inhibited TF and CC infection of target cells ($P<0.05$). ADS-J1 could neutralize the positive charge carried by SEVI in a concentration-dependent manner. Conclusion: SEVI can promote TF and CC virus infection, and ADS-J1 may antagonize SEVI-enhanced TF and CC infection by neutralizing the positive charge on the SEVI surface.

Full Text

Preamble

ADS-J1 Antagonizes Semen-Derived Enhancer of Virus Infection-Mediated Enhancement of Transmitted Founder HIV-1 and Its Matched Chronic Control Strain Infection

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Abstract

Objective
To investigate the effect of semen-derived enhancer of virus infection (SEVI) on transmitted/founder (TF) HIV-1 and its matched chronic control (CC) viruses, and to elucidate the mechanism by which ADS-J1 antagonizes SEVI-mediated enhancement of viral infection.

Methods
Thioflavin T (ThT) assay was used to verify that PAP248-286 can self-assemble into SEVI amyloid fibrils. A pair of TF and CC infectious clone viruses was amplified and mixed with SEVI before infecting TZM-bl cells. Luciferase activity was measured 72 hours post-infection to evaluate the fold enhancement of viral infection by SEVI. To examine the antagonistic effect of ADS-J1, SEVI was pretreated with various concentrations of ADS-J1 before mixing with TF or CC viruses and infecting TZM-bl cells, with luciferase activity again assessed after 72 hours. Additionally, ADS-J1 was directly incubated with viruses to evaluate its direct antiviral activity. Finally, Zeta potential measurements were performed on SEVI treated with different ADS-J1 concentrations to preliminarily explore the underlying mechanism.

Results
ThT assay confirmed that PAP248-286 self-assembles into SEVI amyloid fibrils. SEVI significantly enhanced infection by both TF and CC viruses ($P<0.05$). ADS-J1 not only significantly antagonized SEVI-mediated enhancement of TF and CC infection ($P<0.05$) but also directly inhibited infection of target cells by both viral strains ($P<0.05$). ADS-J1 neutralized the positive charge of SEVI in a concentration-dependent manner.

Conclusion
SEVI promotes infection of TF and CC viral strains, and ADS-J1 likely antagonizes SEVI-mediated enhancement of TF and CC infection by neutralizing the positive surface charge of SEVI.

Keywords: semen-derived enhancer of virus infection; transmitted founder virus; chronic control virus; infection; ADS-J1

Introduction

HIV-1, the etiological agent of acquired immunodeficiency syndrome (AIDS), is predominantly transmitted through sexual contact. Despite being a relatively inefficient route—with unprotected vaginal intercourse carrying an estimated transmission risk of only 1/1000 to 1/10000—approximately 80% of sexual transmission cases result from the establishment of new infection by a single HIV-1 variant, known as the transmitted/founder (TF) virus. Studies have demonstrated that compared to the viral diversity observed in chronically infected individuals, viruses detected during acute infection exhibit high homology, indicating that a "mucosal bottleneck" substantially reduces genetic diversity during transmission. This bottleneck, combined with selective pressure from local innate immune responses in the genital tract, favors the transmission of a limited number of viral variants with enhanced fitness. The viral quasispecies present in chronically infected patients that correspond to these transmitted variants are termed chronic control (CC) viruses. Sequence analyses of acutely infected individuals and their matched chronic controllers have revealed that viruses with more recent ancestral genes are preferentially transmitted, with the consensus sequence representing a portion of the viral genome that initiates infection. Since TF viruses are the strains that establish new infections, blocking TF transmission is essential for preventing HIV-1 spread.

Research by German scientist Professor Münch identified semen-derived enhancer of virus infection (SEVI) as a factor that dramatically enhances HIV-1 infection, substantially increasing the probability of sexual transmission. On one hand, the viral population in semen from infected men comprises only a few strains, and while the highest-titer strains in semen are often not TF viruses, these high-titer common strains coexist with SEVI and both TF and CC viruses in seminal fluid. On the other hand, semen itself plays a critical role as a transmission medium in mucosal HIV-1 spread. Previous studies on SEVI-mediated enhancement of HIV-1 infection have shown that SEVI broadly promotes infection of target cells by CXCR4-tropic, CCR5-tropic, and dual-tropic common strains, though its effects on TF and CC viruses remain unclear.

ADS-J1 is a small-molecule anionic compound identified by our laboratory that inhibits HIV-1 entry into target cells. In this study, we amplified a matched pair of TF and CC clone viruses. We first examined the effect of SEVI on TF and CC infection by mixing SEVI with each virus before infecting TZM-bl cells, then evaluated the direct antiviral activity of the entry inhibitor ADS-J1 against TF and CC viruses and its antagonistic effect against SEVI-mediated enhancement. By measuring the Zeta potential of SEVI after ADS-J1 treatment, we preliminarily explored the mechanism underlying ADS-J1' s antagonism of SEVI-mediated enhancement of TF and CC infection, providing insights into the mechanisms of HIV-1 mucosal transmission.

Materials and Methods

Cell Lines, Viruses, and Reagents

The 293Tx, TZM-bl, and CEMx174 5.25M7 cell lines were obtained from the NIH AIDS Reagent Program. Infectious clone plasmids for CH236 TF and CH236 CC viruses were kindly provided by Dr. Beatrice H. Hahn at the University of Pennsylvania. The peptide PAP248-286 (purity >95%) was purchased from Beijing SciLight Biotechnology and dissolved in PBS at 10 mg/mL for storage at -80°C. RPMI 1640 medium, DMEM medium, trypsin (0.25% EDTA), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco (USA). Thioflavin T was purchased from Sigma-Aldrich. ADS-J1 was synthesized in-house. Phosphate-buffered saline (PBS) was from Guangzhou Geneseed Biotech. PEI transfection reagent was from Shanghai Qifa Biotechnology. Luciferase assay kits were from Promega (USA).

Instruments

CO incubators and centrifuges were from Thermo Fisher Scientific (USA). Biological safety cabinets were from ESCO (Singapore). A Thermomixer comfort was from Eppendorf (Germany). A Genios Pro Tecan microplate reader was used for fluorescence and luminescence measurements. An inverted optical microscope was from Nikon (Japan). Zeta potential was measured using a Zetasizer Nano ZS90 (Malvern, UK).

Experimental Procedures

Thioflavin T (ThT) Assay
PAP248-286 stock solution was diluted to 2.5 mg/mL in PBS and incubated at 37°C with shaking at 1400 rpm for 48 hours to induce aggregation into SEVI amyloid fibrils. SEVI solutions were serially diluted (400, 200, 100, and 50 g/mL) and immediately mixed with ThT for fluorescence detection using a microplate reader (excitation: 450 nm, bandwidth 5 nm; emission: 535 nm, bandwidth 10 nm).

MTT Cytotoxicity Assay
TZM-bl cells were seeded in 96-well plates at 10 cells per well in 100 L volume and incubated overnight at 37°C. The following day, 50 L of fresh DMEM containing 10% FBS was added to each well, followed by 50 L of various SEVI dilutions. After 72 hours of incubation, the medium was removed and 100 L of 0.5 mg/mL MTT solution was added. Following a 4-hour incubation, the supernatant was discarded and 150 L of DMSO was added to dissolve the purple formazan crystals. After 10 minutes of shaking, absorbance was measured at 570 nm. Each concentration was tested in triplicate. A similar protocol was used for CEMx174 5.25M7 suspension cells, which were cultured in RPMI 1640 medium supplemented with 10% FBS, 200 g/mL G418, 1 g/mL puromycin, and penicillin/streptomycin, seeded at 10 cells per well in 150 L volume. After

72 hours, plates were centrifuged at 1500 rpm for 5 minutes, supernatant was removed, and subsequent steps were performed as described for TZM-bl cells.

Virus Production by PEI Transfection

293Tx cells were seeded in 6-well plates at 5×10 cells/mL (2 mL per well) and incubated overnight at 37°C. When cells reached 80% confluence, the medium was replaced with fresh DMEM 2 hours before transfection. CH236 TF or CH236 CC plasmids (4 g per well) were mixed with PEI transfection reagent (1 g/ L) at a 3:1 mass ratio and used to transfect 293Tx cells. After 10 hours, the supernatant was replaced with fresh DMEM containing 10% FBS. Cells were cultured for an additional 48 hours, after which the supernatant (virus stock) was collected, centrifuged, filtered, aliquoted, and stored at -80°C.

Luciferase Reporter Assay

TZM-bl cells were seeded in 96-well plates at 10 cells per well (100 L) and incubated overnight. The following day, CH236 TF or CC viruses were mixed 1:1 with serially diluted SEVI solutions and incubated at room temperature for 10 minutes. The mixture (100 L) was then added to cells. Medium-only and virus-only controls were included. After 3 hours of infection, the inoculum was replaced with fresh DMEM containing 10% FBS. Cells were cultured for 72 hours, then lysed and processed according to the luciferase assay kit protocol: supernatant was removed, wells were washed with 200 L PBS, 50 L of cell lysis buffer was added, and plates were shaken for 20 minutes. Forty microliters of lysate was transferred to white 96-well plates, mixed with 70 L of luciferase substrate (pre-diluted 1:1 with water), and luminescence was immediately measured using a microplate reader. All conditions were tested in triplicate.

To evaluate ADS-J1' s effect on SEVI-mediated enhancement, serial dilutions of ADS-J1 were mixed 1:1 with SEVI, incubated at 37°C for 15 minutes, then centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded, the pellet was resuspended in DMEM, mixed 1:1 with virus, and incubated at room temperature for 10 minutes before infection as described above. For assessing direct antiviral activity, ADS-J1 dilutions were mixed 1:1 with virus, incubated for 10 minutes, and processed similarly. The virus-only control (without ADS-J1) was defined as 0% inhibition and served as the positive control.

Zeta Potential Measurement

ADS-J1 serial dilutions (240, 120, 60, and 30 g/mL) were mixed 1:1 with SEVI (400 g/mL) and incubated at 37°C for 30 minutes. Samples were then centrifuged at 5000 rpm for 5 minutes at room temperature, the supernatant was removed, and the pellet was resuspended in 1 mL of 1 mM KCl. After 10 minutes of incubation at room temperature, samples were transferred to a Zeta potential cuvette and measured using a Zetasizer Nano ZS90. Each sample was measured in triplicate.

Statistical Analysis

Data were processed using Excel and analyzed with SPSS 22.0 software. Results are presented as mean ± standard deviation. One-way ANOVA was used

for statistical analysis, with LSD test for pairwise comparisons when variances were homogeneous, or Dunnett' s T3 test when variances were heterogeneous. Differences were considered statistically significant at $P<0.05$.

Results

PAP248-286 Self-Assembles into SEVI Amyloid Fibrils in PBS

Amyloid fibrils specifically bind thioflavin T, inducing strong fluorescence absorption, whereas peptide monomers do not exhibit this property. ThT assay results demonstrated that PAP248-286 formed amyloid fibrils (SEVI) in PBS in a concentration-dependent manner [Figure 1: see original paper].

SEVI Exhibits No Cytotoxicity in the Tested Concentration Range

One-way ANOVA of MTT assay data from TZM-bl cells revealed homogeneous variances ($P=0.250$). LSD test comparisons between the control group (0 g/mL SEVI) and all treatment groups showed no significant differences ($P>0.05$) [Figure 2A: see original paper], indicating that SEVI concentrations up to 50 g/mL were non-toxic to TZM-bl cells. Similarly, analysis of CEMx174 5.25M7 cells showed homogeneous variances ($P=0.213$), and LSD comparisons revealed no significant differences between the control and treatment groups ($P>0.05$) [Figure 2B: see original paper], confirming that SEVI was non-toxic to CEMx174 5.25M7 cells at concentrations up to 50 g/mL.

SEVI Enhances Infection by CH236 TF and CC HIV-1 Strains

Previous studies have demonstrated that amyloid fibrils formed from different fragments of seminal protein PAP (including SEM1, SEM2, and SEVI) can enhance infection by common HIV-1 strains. Using the TZM-bl reporter system, we examined whether SEVI promotes TF and CC HIV-1 infection. One-way ANOVA yielded P values of 0.091 for CH236 TF (homogeneous variances) and 0.006 for CH236 CC (heterogeneous variances). LSD and Dunnett' s T3 tests, respectively, revealed that both TF and CC viruses showed similar patterns: only the low concentration (3.2 g/mL) group did not differ significantly from the blank control ($P>0.05$) [Figure 3: see original paper], while concentrations of 8, 20, and 50 g/mL showed significant differences ($P<0.05$). These results demonstrate that SEVI enhances TF and CC HIV-1 infection of target cells in a concentration-dependent manner at higher concentrations.

ADS-J1 Antagonizes SEVI-Mediated Enhancement of CH236 TF and CC HIV-1 Infection

ADS-J1 is a small-molecule anionic compound known to antagonize SEVI-mediated enhancement of common HIV-1 strains. Having confirmed SEVI' s enhancing effect on TF and CC viruses, we next set SEVI concentration at 20 g/mL to evaluate ADS-J1' s antagonistic activity. One-way ANOVA

of CH236 TF data showed homogeneous variances (P=0.112), and LSD test revealed that ADS-J1 at concentrations of 0.51, 1.28, 3.20, 8.00, and 20.00 g/mL significantly antagonized SEVI-mediated enhancement of CH236 TF infection (P<0.05) [Figure 4A: see original paper]. For CH236 CC, variances were heterogeneous (P=0.033), and Dunnett' s T3 test showed significant antagonism at ADS-J1 concentrations of 1.28, 3.20, 8.00, and 20.00 g/mL (P<0.05) [Figure 4B: see original paper]. These findings indicate that ADS-J1 antagonizes SEVI-mediated enhancement of TF and CC HIV-1 infection in a concentration-dependent manner.

ADS-J1 Directly Inhibits CH236 TF and CC HIV-1 Infection

The HIV-1 transmembrane glycoprotein gp41 mediates fusion between the virus and target cells, enabling viral entry. Previous research has shown that ADS-J1 inhibits HIV-1 entry by preventing formation of the gp41 six-helix bundle structure. We therefore tested a concentration series of ADS-J1 (0.51, 1.28, 3.20, 8.00, 20.00, and 50.00 g/mL) for its ability to inhibit CH236 TF and CC infection of TZM-bl cells. One-way ANOVA revealed homogeneous variances for both CH236 TF and CC groups (P=0.117 and P=0.061, respectively). LSD pairwise comparisons demonstrated that ADS-J1 at all tested concentrations significantly inhibited infection by both CH236 TF and CC strains (P<0.05) [Figure 5: see original paper]. These results confirm that ADS-J1 not only antagonizes SEVI-mediated enhancement but also directly inhibits TF and CC HIV-1 infection.

ADS-J1 Neutralizes the Positive Charge on SEVI

Since ADS-J1 itself inhibits TF and CC infection, we questioned whether the reduced enhancement observed in [Figure 4: see original paper] represented simple additive effects or true antagonism of SEVI. The mechanism of SEVI-mediated enhancement involves its high positive charge binding to negatively charged viral particles, reducing electrostatic repulsion between virions and cell membranes and thereby increasing viral attachment and fusion. As a small-molecule anionic compound, ADS-J1 might neutralize this positive charge. To investigate this mechanism, we measured the Zeta potential of SEVI treated with various ADS-J1 concentrations. SEVI alone (400 g/mL) exhibited a potential of +20.9 ± 0.36 mV, while ADS-J1 alone (30 g/mL) showed -4.2 ± 0.20 mV. Treatment with ADS-J1 concentration-dependently neutralized SEVI' s positive charge, even reversing it to negative at 60 g/mL ADS-J1 [Figure 6: see original paper]. These results demonstrate that ADS-J1 neutralizes the positive surface charge of SEVI.

Discussion

Sexual transmission has become the predominant route of HIV-1 infection, with genital, rectal, and oral mucosal tissues serving as primary sites of viral entry.

The strains that establish mucosal infection are TF viruses, with approximately 80% of cases resulting from single-variant transmission. During secretion from blood to semen in chronically infected men, both viral load and diversity are substantially reduced, yet some strains persist in semen and can be transmitted. This indicates that the mucosal bottleneck and innate immunity cannot completely prevent transmission but rather select for TF variants capable of overcoming these barriers. Seminal SEVI, present at concentrations up to 42 mol/L and comprising approximately 35% of seminal protein content, significantly enhances HIV-1 infection and likely facilitates TF transmission. Currently, no vaginally-targeted agents have been approved to prevent HIV-1 transmission. Topical microbicides represent a promising approach, potentially acting by directly inactivating HIV-1, blocking viral adhesion, inhibiting viral entry, or enhancing natural mucosal immunity. However, many microbicides effective in vitro have failed in clinical trials, possibly due to SEVI-mediated enhancement.

In this study, we first demonstrated that PAP248-286 peptide monomers form amyloid fibrils (SEVI) in PBS when agitated vigorously at 37°C for 48 hours, as confirmed by ThT assay. While this method produces SEVI for experimental use, the precise mechanisms of SEVI formation in semen remain unclear, potentially involving peptide-peptide interactions, medium hydrophobicity, temperature, pH, and metal ions.

SEVI' s cationic properties enable it to capture negatively charged virions like a "net," reducing electrostatic repulsion and increasing viral attachment to cell membranes. Although TF viruses have lower glycosylation, potentially facilitating mucosal penetration and reducing SEVI capture, their envelope glycoprotein content is nearly double that of common HIV-1 strains, making them particularly susceptible to SEVI-mediated enhancement. Using CH236 TF and CC infectious clones, we demonstrated that SEVI enhanced TF and CC infection by 11.6±0.7-fold and 8.9±0.7-fold, respectively—somewhat weaker than enhancement of common R5 strains, possibly due to differences in intrinsic infectivity and viral titers, which are typically several orders of magnitude lower for TF/CC viruses.

ADS-J1, identified through high-throughput screening in our laboratory, exhibits "triple-action" activity: inhibiting SEVI formation, degrading mature SEVI, and blocking viral entry by targeting gp41/gp120, making it a promising lead compound for SEVI-targeted microbicides. Building on this foundation, we hypothesized that ADS-J1 would antagonize SEVI-mediated enhancement of TF/CC infection. Indeed, pre-incubation of SEVI with ADS-J1 before mixing with TF or CC viruses significantly reduced SEVI' s enhancing effect in a concentration-dependent manner. High ADS-J1 concentrations reduced viral titers below control levels, suggesting direct antiviral activity, which we subsequently confirmed. Since SEVI' s positive charge is critical for its enhancing activity and ADS-J1 is anionic, we investigated charge neutralization as the mechanism. Zeta potential measurements confirmed that ADS-J1 concentration-dependently neutralized SEVI's positive charge, reversing it to negative at higher

concentrations. Thus, ADS-J1 antagonizes SEVI-mediated enhancement of TF and CC infection through the same charge-neutralization mechanism observed with common R5 strains.

Using an in vitro infection model, we have demonstrated ADS-J1' s ability to antagonize SEVI-mediated enhancement of TF and CC infection and elucidated its mechanism. Given that TF viruses are the strains that effectively establish new infections, these findings provide more relevant insights into mucosal transmission than studies using common laboratory strains. However, due to the complexity of the in vivo environment, further studies using animal models or systems that better recapitulate physiological conditions will be necessary to fully understand TF transmission mechanisms and develop effective strategies to block TF infection.

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Note: Figure translations are in progress. See original paper for figures.