Antiviral Research 82 (2009) 22–28

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Retroviral self-inactivation in the mouse vagina induced by short DNA

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ARTICLE INFO

Article history:
Received 30 April 2008
Received in revised form
22 December 2008
Accepted 13 January 2009

Keywords:
Lentiviral vector
HIV
RNase H
Microbicide
Virus suicide
Hairpin-loop DNA

ABSTRACT

Human immunodeficiency virus (HIV) has been shown to undergo self-destruction upon treatment of cell-free virions with partially double-stranded oligodeoxynucleotides targeting the polypurine tract (PPT) of the viral RNA in the virus particle. The ODN forms a local hybrid with the PPT activating the viral RNase H to prematurely cleave the genomic RNA. Here we are describing the self-destruction of a recombinant lentivirus harboring the PPT of HIV in a mouse vagina model. We showed a decrease in viral RNA levels in cell-free virus particles and a reduction reverse transcribed complementary DNA (cDNA) in virus-infected human and primary murine cells by incubation with ODNs. In the vagina simultaneous, prophylactic or therapeutic ODN treatments led to a significant reduction in viral RNA levels. Our finding may have some relevance for the design of other viral self-destruction approaches. It may lead to a microbicide for reduction of sexual and mother-to-child transmission.

1. Introduction

We have previously demonstrated that human immunodeficiency virus (HIV-1) can be in activated in vitro and rendered non-infectious by an oligodeoxynucleotide (ODN). The ODN A is a partially double-stranded 54-mer hairpin-loop-structured DNA designed to bind specifically to the polypurine tract, PPT, of HIV-1, a highly conserved region of the HIV genome. The DNA forms a local RNA–DNA hybrid with the viral RNA genome and mimics a natural replication intermediate (Jendis et al., 1998, 1996; Moelling et al., 2006). The hybrid is a substrate for the RT/RNase H (Moelling et al., 1971) and is specifically cleaved to generate the primer for the (+) strand DNA synthesis (Wöhrl and Moelling, 1990). The RT/RNase H forms a heterodimer, which is located inside the virus particles and then carried into the cell during infection. Therefore the inhibitory action of ODN A can be exerted already in the viral particles before they infect the cell (Matskevich et al., 2006).

Recently, we demonstrated in a retrovirus mouse model that an ODN that targets the PPT of the retrovirus can reduce disease progression, prevent infection or reduce the viral load in the blood (Matzen et al., 2007). However, this murine oncogenic retrovirus model does not reflect the situation of sexual HIV transmission. Therefore we tested an application of ODN against a lentiviral vector applied to the mouse vagina as a model for sexual transmission. The lentiviral vector FUGW (Flap, ubiquitin promoter, GFP and WRE vector) contains the HIV–PPT sequence, which is essential for its single replication round. Here we demonstrate that FUGW present in the mouse vagina or in human vaginal or cervical cell lines can be treated with ODN A. We applied chemical modifications of the ODN such as phosphorothioates or 2′-*O*-methyl groups to protect against nucleases and increase stability. In all cases, statistically significant reduction of FUGW virus RNA copies was observed with ODNs compared to their respective randomized sequence serving as negative controls. The approach we are using differs from previous ones, since it is based on the activation of a retroviral enzyme for destruction of the virus, instead of inhibition. This may be worth developing further.

2. Materials and methods

2.1. Oligodeoxynucleotides

All oligodeoxynucleotides (ODNs) were phosphorothioated at each end and in the T4 linker and have the same length (54-mer). ODN A consists of an antisense strand of PPT and a passenger strand. ODN AM is the methylated version of the ODN A (Fig. 1A). ODN S2 consists of a randomized sequence of both strands serving as a control for non-specific activity of ODN A. ODN S2 M is therefore the methylated sequence of the ODN S2 (Fig. 1A). The ODNs were purchased from Operon or Integrated DNA Technology.

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doi:10.1016/j.antiviral.2009.01.002

2.2. Lentiviral vector production

FUGW is a lentiviral self-inactivating vector with a VSV-G coat, which allows transduction of many cell-types but does not replicate. This lentiviral vector contains the PPT sequence that is identical to that of HIV. For generating high titer lentiviral vectors, 7×10^6 human embryonal kidney (HEK) 293T cells in 10 cm plates were co-transfected with cFUGW, pCMVR8.9, and pHCMV-G (5 μg each) using lipofectamine 2000 (Invitrogen). Six hours post-transfection 8 ml fresh medium replaced the original medium. Two days post-transfection the culture medium was collected and filtered through a 0.45 μm filter. The filtrated medium was then ultracentrifuged twice for 90 min using Polyallomer tubes. Titers of viral preparations were assessed by infecting 5×10^5 C81–66 cells with serial dilution of the virus and determining the fraction of GFP-positive cells 1 day after infection by FACS. Titers of $5–9 \times 10^7$ transducing units (TU) per ml were routinely obtained.

2.3. RT/RNase H cleavage assay

HIV–PPT RNA was in vitro transcribed, dephosphorylated and 5′-phosphorylated as described (Matskevich et al., 2006). Different ODNs (10 nM) and HIV–PPT RNA (10 nM) in RT buffer were annealed (2 min 90 °C, 10 min 37 °C) and cleaved with 0.1 U/μl RT/RNase H (GE Healthcare, Piscataway, NJ) for 30 min at 37 °C. The samples were subjected to electrophoresis in 10% polyacrylamide containing 8 M urea, together with HIV–PPT RNA partially digested with RNase T_1 (Ambion) as described by the manufacturer.

2.4. Virions studies

FUGW virions (10^4 TU) were either treated with different concentrations of ODN A (0–25 μM) for titration or different ODNs for 4 h at 37 °C. These virions were also titrated using either 10^4 IU or 10^5 IU with 5 μM ODN A for also 4 h at 37 °C. Viral RNA was then extracted using the viral RNA mini kit (Qiagen) and eluted with 50 μl elution buffer. Then 5 μl RNA was used for cDNA synthesis in a reaction volume of 12.5 μl and 5 μl of the cDNA was used for qPCR.

2.5. In vitro studies

Both cell lines VK2/E6E7 and Ect1/E6E7 were purchased from the American Type Culture Collection (ATCC). The VK2/E6E7 (ATCC CRL-2616) cell line was established from normal vaginal mucosal tissue taken from a premenopausal woman undergoing anterior–posterior vaginal repair surgery. The ectocervical Ect1/E6E7 (ATCC CRL-2614) cell line was established from normal epithelial tissue taken from a premenopausal woman undergoing hysterectomy for endometriosis (Fichorova et al., 1997). These two cell lines were seeded in a 24-well-plate at a density of 1×10^5 cells/well in Keratinocyte-Serum Free medium (Invitrogen) supplemented with 0.1 ng/ml human recombinant epidermal growth factor (EGF, Sigma–Aldrich), 0.05 mg/ml bovine pituitary extract (Invitrogen), and additional 44.1 mg/ml calcium chloride (final concentration 0.4 mM). Cells were infected with 10^4 IU of FUGW resulting in a multiplicity of infection (MOI) of 0.1, for 2 days at 37 °C. Cells were washed twice with PBS and then genomic DNA (gDNA) was extracted using the blood DNA mini kit (Qiagen) and eluted in 30 μl elution buffer. For qPCR analysis, 100 ng of total gDNA was used in a reaction volume of 25 μl. Primary vaginal cells extracted from mouse vaginal lavage fluid were cultured in epithelial cell medium (ECM) as described (Macartney et al., 2000). ECM consists of equal volumes of phenol-red-free DME (Sigma–Aldrich) and Ham's F-12 medium (Invitrogen) supplemented with 10% FCS, 100 mg/ml streptomycin, 100 IU/ml penicillin, 1 mmol/ml L-glutamine, and 10 ng/ml EGF. The vaginal cells at a density of 5×10^4 cells in 500 μl volume were infected with 10^4 FUGW TU corresponding to a MOI of 0.2 for 1 day at 37 °C. Cells were washed twice with PBS and then gDNA was extracted and eluted in 30 μl elution buffer. For PCR, 40 ng of total gDNA was used in a reaction volume of 25 μl.

2.6. Mouse model and FUGW virus challenge

Six- to eight-week old female C57BL/6 mice purchased from Harlan (Zeist) were used throughout these studies. Animal studies were performed according to Swiss Animal Rights in the animal facilities of the Institute of Medical Virology, University of Zurich, with permission by the Zurich-Veterinary-Office (213/00). Mice were kept in conventional conditions with full access to food and water. All mice were treated s.c. with 2.5 mg of progestin (Depo-Provera, Pfizer) to synchronize the estrus cycle (Khanna et al., 2002). One week later, the progestin-treated mice were anesthetized with isoflurane (ABBOTT AG) and challenged with an intravaginal inoculum of 20 μl 3% carboxymethyl cellulose sodium (CMC) medium (Roberts et al., 2007) (Sigma–Aldrich) containing 10^4 IU FUGW with or without 25 μM ODNs. Four hours later the mice were euthanized and vaginal lavage with 100 μl of sterile PBS was performed. Viral RNA was then extracted from these vaginal lavage fluids using the viral RNA mini kit (Qiagen).

2.7. Detection of FUGW by Real-Time PCR

For qRT-PCR RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the Manufacturer's instructions.

Primers and Probes flanking the PPT sequence of FUGW were: 5′-GAGGAGGTGGGTTTTCCAGT-3′ (forward), 5′-GGGAGTGAATTAGCCCTTCC-3′ (reverse), and FAM-5′-ACCTTTAAGACCAATGACTTACAAGGCAGC-3′-TAMRA (probe); for mouse glyceraldehyde-3 phosphate dehydrogenase (mGAPDH), 5′-CTTCACCACCATGGAGAAGGC-3′ (forward), 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse). FAM-5′-CCTGGCCAAGGTCATCCATGACAACTTT-3′-TAMRA; for human glyceraldehyde-3 phosphate dehydrogenase (hGAPDH), 5′-GTTCCAATATGATTCCACCC-3′ (forward), 5′-GAAGATGGTGATGGGATTTC-3′ (reverse), FAM-5′-CAAGCTTCCCGTTCTCAGCCTAMRA-3′ (probe). All primers and probes were purchased from Microsynth. The cycling conditions were 50 °C for 2 min (1 cycle), 95 °C for 10 min (1 cycle), 95 °C for 15 s and 60 °C for 1 min (50 cycles). The results are presented in the figures as FUGW RNA or DNA corresponding to absolute copy numbers per assay.

2.8. Statistical analysis

The statistical significance of the antiviral activity of ODN A and ODN AM in virions and in vitro was determined by Student's *T*-test and the results were expressed as mean ± SEM (error bars in graph). These *P*-values were for two-tailed significance test. For the in vivo studies, viral RNA levels after treatment were compared by analysis of variance with Bonferroni post-hoc test applied to logarithmically transformed and PBS normalized data using SPSS 13.0 (SPSS Inc., IL). Differences were considered to be significant at $P < 0.01$.

3. Results

3.1. Analysis of ODNs and lentiviral system

In order to establish an animal model for the antiviral activity of ODN we used the lentiviral vector FUGW (Fig. 1A). FUGW is a lentiviral self-inactivating vector, which carries a green fluorescent protein, GFP, reporter driven by an internal ubiquitin promoter.