Anal intraepithelial neoplasia in HIV+ men
Richel, Olivier

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Increased HIV-1 activity in Anal Intraepithelial Neoplasia foci compared to unaffected anal mucosa in MSM

Georgios Pollakis, Olivier Richel, Joost D Vis, Jan M Prins, William A Paxton, Henry JC de Vries

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Abstract

HPV related anal carcinoma is an increasing health problem amongst HIV-1 positive individuals. We studied 3 patients with focal intra-anal high grade anal intraepithelial neoplasia (HGAIN, AIN-3). In all 3, higher levels of HIV-1 RNA and DNA were detected in HGAIN lesions compared to healthy mucosal tissue. In 2 patients, HIV-1 episomal DNA could be found in CD4+ and CD14+ fractions in plasma, indicative of ongoing viral replication. Furthermore, in 1 patient we found HIV-1 episomal DNA in HGAIN lesions but not healthy mucosa. Our findings indicate heightened HIV-1 activity in anal HGAIN lesions, not in healthy neighboring anal mucosa.
Introduction

there is increasing evidence that successful HIV-1 antiretroviral therapy (ART), with sustained undetectable plasma RNA load, does not completely suppress residual virus replication.¹ The continual reseeding of cellular reservoirs with virus, especially within multiple sequestered anatomical sites, has major implications when considering efforts aimed at eradication of HIV-1 infection. Co-infection with other pathogens might influence both the dynamics of viral reservoirs and the shedding of HIV-1 at sites of inflammation. Amongst HIV-1 positive men having sex with men (MSM) up to 95% have been reported with anal HPV infection.²,³ Anal HPV infection is independently associated with HIV acquisition.⁴ Reversely, the rate of HPV diagnosis was shown to increase in a group of women from South Africa during their first year of HIV-1 infection, indicating mucosal immune dysfunction from the early stages of HIV-1 disease.⁵ Infection with HPV is considered to be a risk for the induction of anal cancer in MSM.⁶ Invasive anal cancer is preceded by mucosa abnormalities known as high grade anal intraepithelial neoplasia (HGAIN). Unlike most other opportunistic infections, the introduction of ART has not reduced the rates of diagnosed anal cancer.⁷ Longer life expectancy of ART treated individuals, with an already impaired immune system, could be one possible contributing factor.⁸ It has been shown that HIV-1 positive individuals are less prone to clear their HPV infection compared to uninfected individuals.⁹ In addition, higher HIV-1 viral shedding was reported among both men and women co-infected with HPV.⁸ Prolonged HPV infection of the anal mucosa will result in inflammation that will undoubtedly increase the numbers of immune cells both carrying HIV-1 as well as uninfected cells with heightened susceptibility for infection. Here we aimed to address whether HPV infection can influence HIV-1 burden at the anal mucosa.

Methods

This study was reviewed and approved by the Medical Ethical Committee of the Academic Medical Centre, University of Amsterdam, Netherlands. After written consent, HIV-positive MSM were included with recently diagnosed focal HGAIN (AIN-3) lesions as described elsewhere.⁹ Under high resolution anoscopy...
2 adjacent biopsies were taken from a suspected AIN lesion and 2 adjacent biopsies from unsuspicious normal appearing anal mucosa. From each location 1 biopsy was processed for histopathology to confirm AIN and normal mucosa, respectively. The other 2 biopsies from respective locations were snap frozen in liquid nitrogen and cryopreserved at -150°C. At time of sample collection, plasma HIV-1 RNA was measured using the Abbott Molecular, m2000 Real-Time System (DES PLAINES, Ill. and ROCKVILLE, Md.). CD4+ T-lymphocyte count was carried out with standard fluorescent activated cell sorting (FACS), using commercially available fluorescent-labeled antibodies (Becton-Dickinson Immunocytometry, CA, USA). Additionally, approximately 20 ml blood was drawn in BD Vacutainer® CPT™ (with sodium citrate) cell preparation tubes the same day as the biopsies were collected. PBMCs were isolated from the blood and cryopreserved (-150°C) as was 10 ml of plasma (stored -80°C).

Thawed tissue was fully digested with proteinase K (Roche Applied Science) at 56°C overnight. In order to prevent RNA loss the proteinase K deactivation step at 95°C was skipped following treatment and was replaced by a nucleic acid isolation procedure using the “QIAamp Viral RNA kit” (QIAGEN). The MACS cell separation system was used to isolate CD14+ monocytes and CD4+ lymphocytes from PBMC followed by nucleic acid isolation utilizing the Boom method. The plasma was ultra centrifuged (2 hours at 32,000 rpm) after which the pellet was collected for nucleic acid isolation again utilizing the Boom extraction method.

Nucleic acid detection was performed by an ultrasensitive protocol using highly conserved primers and probe located within the RU5 long terminal repeat region of the viral genome and which was performed by the QIAGEN OneStep RT-PCR Kit according to instructions¹⁰. This assay detects all forms of viral RNA and DNA, however, a pre-amplification step and choice of specific oligonucleotide primers allows for differentiation between linear and circular viral forms. The pre-amplification primers {5’GAGCCTGGGAGCTCTCTGGCTA/5’AGCAAGCCGA GTCTGCATGC} recognize all forms of RNA and DNA while {5’TACCCTCAGATGCTGACA/5’AGCAAGCCGAAGTCCTGCGTC} only amplify fully reverse transcribed HIV-1 DNA. Linear versus circular or integrated versus non-integrated viral DNA could not be distinguished by this assay; however, including a DNAse incubation step (Ambion® TURBO™ DNAse from Life Technologies) we could differentiate between RNA as well as both RNA and fully reverse transcribed viral DNA. Episomal HIV-1 DNA was pre-amplified with the primer-pair {5’GAGCCTGGGAGCTCTCTGGCT/5’TCCACAGATCAAGGATATCTTGTC}. The tissue
isolate input in the amplification reaction was expressed in mg of tissue with equal tissue input quantities utilized in all experiments.

Table 1 Characteristics of participants

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>AIN grade</th>
<th>Present CD4+ Cell count (cells/10^6/ml)</th>
<th>Nadir CD4+ Cell count (cells/10^6/ml)</th>
<th>HIV-RNA (copies/ml)</th>
<th>Antiretroviral therapy (ART)</th>
<th>Duration of ART (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AIN 3</td>
<td>610</td>
<td>180</td>
<td>&lt;40</td>
<td>Atripla</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>AIN 3</td>
<td>580</td>
<td>190</td>
<td>&lt;40</td>
<td>Truvada atazanavir</td>
<td>14.1</td>
</tr>
<tr>
<td>3</td>
<td>AIN 3</td>
<td>718</td>
<td>320</td>
<td>&lt;40</td>
<td>Truvada atazanavir ritonavir</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Results

Three individuals previously diagnosed with AIN 3 were included in the study. All were receiving successful ART, as evidenced by an undetectable plasma RNA load, and their CD4+ cell-counts had recovered to relatively high numbers (Table 1). In all 3 participants AIN 3 could be reconfirmed by histopathology upon inclusion. Biopsies from normal appearing mucosal lining showed normal differentiation and no signs of neoplasia. First, we used a sensitive PCR (described in 10) that amplified a 122nt LTR HIV-1 DNA fragment from fully reverse transcribed viral RNA to detect virus in the leukocyte fractions. Both CD14+ and CD4+ fractions were positive for viral DNA with the exception of the CD14+ cell fraction from patient 3 (data not shown). Virus RNA was then investigated in the plasma as indication of de-novo virus production. With our sensitive PCR assay we could detect fully reverse transcribed viral DNA in all three study participants, indicating that the ultracentrifugation of the plasma had pelleted DNA-containing cell-debris (Fig. 1A). However, in order to avoid HIV-1-DNA contamination from infected cells during RT-PCR, the assay input was subjected to a DNAse I treatment prior to reverse transcription. The amplification procedure under these conditions revealed the presence of
HIV-1 RNA in the plasma samples of patients 2 and 3 but not patient 1 (Fig. 1A), indicating that virus is indeed replicating within the blood compartment, shedding virus in the plasma.

We next determined whether HIV-1 RNA or DNA could be detected in the anal mucosa despite successful ART. We analyzed in parallel the healthy and lesional mucosa samples from all 3 study participants. Here the choice of oligonucleotides (and depending on whether the sample was treated with DNase or not) enabled us to detect viral RNA and DNA or alternatively RNA alone (Fig. 1B). For all 3 study participants we found HIV-1 RNA and DNA in both healthy and lesional mucosal tissues and using density quantification of the amplified product, we found higher quantities of RNA in the lesional HGAIN biopsies for all subjects (Fig. 1C). Utilizing tissue from patient 1 (from whom sufficient material was available) we performed a quantitative TaqMan assay utilizing the same conditions and again identified higher levels of both HIV-1 RNA and DNA in lesional AIN versus healthy mucosal tissue (Fig. 1D).

The presence of HIV-1 RNA does not allow us to conclude whether the de-novo produced virus originates from recently or historically quiescently infected cells nor indicate the presence of infectious virus particles. We addressed this by testing for the presence of episomal HIV-1 DNA, which is indicative of recently infected cells. We found HIV-1 episomal DNA in the PBMC from patients 1 and 2 in both the CD14+ and CD4+ cell fractions. Furthermore, from patient 2, diagnosed as HIV-1 positive since 1995 we found episomal DNA in the lesional HGAIN biopsy whilst the healthy mucosa sample was negative (Fig. 1E), indicating both increased viral activity and the presence of recently infected cells in the lesion.

**Discussion**

It is well established that there is interplay between HIV-1 and HPV viruses, since infection by one is associated by increased infection rates of the other. HIV-1 infected individuals do not clear their HPV infection despite suppressive antiretroviral therapy and HPV/HIV-1 positive individuals shed higher levels of HIV-1 at the anal mucosa than those who are HIV-1 positive alone.\(^2\)\(^5\) We have shown here increased levels of HIV-1 viral RNA in HGAIN intra-anal lesions compared with the adjacent healthy mucosa. This could provide one explanation
why HIV-1/HPV co-infected individuals shed higher levels of HIV-1 in their anal mucus. Although our study sample is small, the presence of higher levels of HIV-1 RNA in HGAIN lesions compared to normal adjacent mucosa in all 3 participants is an indication that neoplastic lesions are indeed associated with viral activity. This could be caused by increased virus replication due to heightened T cell activation locally, or alternatively, by the recruitment of infected T cells (infected with HIV-1) to the lesional tissue. We found both increased viral RNA as well as DNA load in HGAIN lesions, as indication of increased local viral activity. We do not know how ever whether it is due to the local immune activation or the recruitment of virus producing cells at the site of inflammation.

Since all study participants were under suppressive ART for as long as 15 years, the presence of HIV-1 RNA in AIN lesions clearly supports the concept that continuous virus replication is ongoing. Zucherman et al. could measure among MSM higher levels of HIV-1 RNA in rectal mucosa secretions than in blood and in seminal plasma, whether they received ART or not. Here we provide further evidence that antiretroviral therapy may not be able to completely suppress HIV-1 replication and that this can occur in rectal mucosal tissue and even more so in AIN lesions where localized inflammation is likely ongoing.

The local presence of HIV-1 RNA does not allow us to conclude whether the de-novo produced virus originates from recently or historically quiescently infected cells nor indicate the presence of infectious virus particles. The viral RNA could be produced either by a reactivated long lived latently infected reservoir or by recently infected cells that ART failed to protect. Episomal HIV-1 DNA is believed to have a short half-life suggesting recent infection. It is important to note that 1 of the patients has received therapy for over 14 years with plasma viral loads consistently below 40 copies/ml. We found episomal DNA both in peripheral CD4+ lymphocytes and CD14+ monocytes, suggesting that the de novo produced HIV-1 particles are infectious despite successfully suppressive ART. We also found episomal DNA in the lesional HGAIN biopsy of one of the patients.

Cage et al. have shown that supernatant from HPV-associated cervical cells could induce HIV-1-p24 and reduced IL-6 production, implying that HPV infection can affect HIV-1 pathogenesis. Increased levels of immunoregulatory cytokines were also associated with HPV infection at the genital site suggesting increased immune activation and inflammation in women co-infected with HPV/HIV.
Pro-inflammatory cytokines may play a key role in the HIV life cycle, especially at the level of gene transcription, favoring the ability of HIV to establish latent reservoirs.

The 3 patients described here all showed increased HIV-1 activity in HGAIN lesions in comparison to non-lesional anal mucosa. Cell populations typically targeted by HIV-1 may be recruited to the infection site through alteration of the local cytokine/chemokine environment such as (MIP)-3a, IL-7, MIP-1B, IL-8 and RANTES, all of which are known to affect HIV-1 infection and replication. It is also possible however that the lesion represents an anatomical site where drug concentrations are lower and where residual HIV-1 replication is increased. An increase in the concentrations of HIV-1 in HGAIN lesions will most likely lead to an increased transmission risk of HIV via receptive anal intercourse. Although the complex interaction of other pathogens with HIV-1 is not well understood at the level of the anal mucosa, the interaction between the viruses and the resultant induced immune responses may play a critical role in influencing HIV-1 replication and/or transmission.
Figure 1:

A: HIV-1 LTR amplification in Plasma of patients 1, 2 and 3 with or without DNAse I in order to distinguish RNA from DNA. When DNAse is added (+), right panel only RNA is detected.

B: HIV-1 LTR amplification from anal mucosa healthy tissue (M) and lesional AIN biopsies (L). Oligonucleotides able to amplify all forms of RNA and DNA (left panel) will always amplify the virus, however, in the presence of DNAse only RNA will be detected. With Oligonucleotides able to amplify only fully reverse transcribed DNA (Only Viral DNA) in the presence of DNAse only DNA will be detected while RNA remains undetectable. With Oligonucleotides able to amplify both viral RNA and DNA, in the presence of DNAse only RNA will be detected (1, 2 and 3 indicate the three study subjects).

C: HIV-1 RNA comparison between the healthy tissue (M) and the AIN lesion (L). The HIV-1 LTR RNA was amplified as described in panel B prior to quantification. RNA amplified from the lesion was set at 100% (black bars) and compared to the amount found in the adjacent healthy area of the mucosa (grey bars).

D: Real time TaqMan quantification of HIV-1 LTR DNA and/or RNA from the anal mucosa of patient 1. The nucleic acids were subjected to a 15 cycle preamplification with oligonucleotides able to amplify either all forms of RNA and DNA or fully reverse transcribed DNA alone. The healthy tissue (M) is shown in grey and the lesion (L) in black bars.

E: Episomal HIV-1 DNA amplification from patient 2. The CD4+ lymphocyte and CD14+ monocyte PBMC fractions were analyzed along with the lesional and healthy areas of the anal mucosa. DNA equivalents of 2.5x10^5 cells were used for the amplification from the blood fractions and of 400 micro-grams of tissue for the amplification from the anal mucosa.
References