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Immunologic, Virologic, and Pharmacologic Characterization of the Female Upper Genital Tract in HIV-infected women

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Abstract

A comparative analysis of cellular and soluble markers of immune activation in HIV-infected women on combination antiretroviral therapy (cART) showed that the upper (UGT) compared to the lower female genital (LGT) tract was characterized by higher frequencies of potential HIV target cells and increased inflammatory molecules. Despite the activated UGT milieu, HIV RNA could not be detected in paired samples of plasma, cervicovaginal (CVL) or endometrial lavage (EML). As ARV concentrations were ≥3 fold higher in the endometrium than the in the lower genital tract, high ARV penetration and/or metabolism may limit viral replication in the UGT.

Keywords

HIV; female; uterus; tenofovir; pharmacokinetics; inflammation

INTRODUCTION

The relationship between elevated blood plasma HIV viral load and increased risk of sexual and vertical transmission is well documented.[1,2] However, viral shedding persists in the female genital tract despite the suppression of plasma viral load even in women taking combination antiretroviral therapy (cART).[3–7] Therefore, blood plasma viral load is not a reliable surrogate marker for viral activity or HIV infectivity in the female genital tract.

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Most mechanistic research on heterosexual transmission of HIV has focused on the vagina and cervix [lower genital tract (LGT)]. [8–18] However, the uterine endometrium [upper genital tract (UGT)] contains activated CCR5+ T-cells, which are a target for HIV transmission.[19] It is plausible that the UGT could serve as site of HIV acquisition, replication, and viral shedding.[20–27] This feasibility study was conducted to better understand the biology of HIV in the UGT.

METHODS

Study Participants

This study was approved by the University of North Carolina Institutional Review Board. We enrolled 16 HIV-1-positive menstruating women, aged 25–48 years, who were fully suppressed by cART with plasma HIV RNA ≤ 40 copies/mL for at least 6 months. Ten HIV-negative women were enrolled to pilot the study procedures, and 4 were enrolled as immunologic study controls. Exclusion criteria included (i) pregnancy or planning pregnancy, (ii) unwillingness to use 2 forms of contraception (condoms + hormonal) or previous bilateral tubal ligation procedure, (iii) irregular menses (not between 21–42 days), (iv) untreated cervical infection (*N. gonorrhea, C. trachomatis*), (v) intrauterine device in place, or (vi) use of immunosuppressive medications. Study samples were taken in the secretory phase of the menstrual cycle based on reported menstrual history.

Specimen Collection

Cervicovaginal fluid (CVF) was collected as described.[28,29] Cervicovaginal lavage was collected using 10 mL phosphate-buffered saline and spun to remove cells.[4] Endometrial lavage (EML) specimens were collected using a Goldstein sonohysterography cathether passed through the internal cervical os, inflated with 1.5 mL of air, and 8 mL of normal saline advanced and withdrawn. Cells recovered from EML were stained for flow cytometric analysis. Endometrial biopsy (EMB) specimens were collected via a 3mm plastic pipelle following EML collection. The EMB was snap frozen in liquid nitrogen and stored at −80ºC; cells were isolated as described for mucosal tissues.[30] Whole blood was collected in EDTA tubes with plasma isolated and stored at −80°C.

Measurement of immune activation

Cellular activation was assessed by staining mononuclear cell suspensions with antibodies to CD3, CD4, CD8, CCR5, CD69, and Ki67 in all participants. Naïve, effector, and memory T cells were characterized by CD45RA and CCR7 in HIV-infected women. Samples were stained as described,[30] acquired on a LSRII instrument and analyzed using FlowJo software (FlowJo, Ashland, OR).

Soluble mediators in CVL and EML were analyzed in all participants using the Human Cytokine and Chemokine Panel I Kit (Millipore, Billerica, MA) using a MagPix instrument, or by ELISA (sCD163: Trillenium Diagnostics, LLC, Brewer, ME; sCD14: and R&D Systems, Minneapolis, MN). CVL specimens contained predominantly sloughed dead epithelial cells, and therefore, no further cellular evaluation was performed.

HIV RNA testing

HIV RNA was measured in plasma, EML and CVL using the Abbott RealTime HIV-1 Assay, and in EMB by real-time PCR[31] after extraction with the RNeasy Fibrous Tissue Mini Kit (Qiagen). Lower limit of quantification was 40 copies/mL.

Quantification of antiretroviral concentrations

Plasma, CVF, and endometrial tissue concentrations of tenofovir (TFV) and emtricitabine (FTC) were measured using previously published liquid chromatography-tandem mass spectrometry (LC-MS/MS) and LC-UV methods.[32–34] Samples below the limit of detection (BLD) were reported as 1/2 the lower limit of quantification (LLOQ). Analyte concentrations for both parent and intracellular metabolite are reported as nanomolar (nM). Solid matrices were converted to nM units assuming a tissue density of 1 g/ml.

RESULTS

Study population

Data were obtained on 12 of 16 HIV-1 positive women who participated in the study. Two participants came for more than one visit. Five samples were not evaluable due to cervical stenosis (n=2) or vaginal bleeding (n=3). Median age of participants was 39 years (range 25–44); 73% of women self-identified as Black or African-American and 27% were Caucasian. All women were either sexually abstinent (56%) or had undergone tubal ligation procedures (44%). The 4 HIV-negative women sampled for immunologic studies were all Caucasian with a median age of 26 years (range 20–42). These women used either oral contraceptive pills plus condoms (75%) or had a prior tubal ligation (25%) for contraception. Participants were taking TFV containing regimens described in Table 1 as well as etravirine/raltegravir/darunavir/ritonavir and atazanavir/ritonavir/abacavir/ lamivudine regimens.

Immune activation of the UGT

Cellular immune activation was measured in EML and EMB cells (n=10). Both EML and EMB specimens contained high frequencies of activated CD4+ and CD8+ T cells. Despite great variation from one woman to another, within the same patient, activation in EML and EMB samples were comparable. Frequencies of potential HIV target cells, CCR5-positive CD4+T cells, ranged from 9.7% to 74.3% and from 2.4 to 72.3% in EML and EMB samples, respectively (Figure 1A). Similarly, a large percentage of CD4+ T cells expressed the activation marker CD69 (Figure 1A). As >80% of T cells expressed markers typical for memory T cells (data not shown), CD69+ cells likely represented tissue resident memory T cells. This conclusion was supported by the apparent lack of proliferation (Ki67) among CD4+ and CD8+T (Figure 1A).

To determine whether increased immune activation in the upper FGT was the result of HIV infection, or represented a normal physiologic condition, we analyzed samples from 4 HIV-uninfected women. Of note, there was no EMB sample from one participant and 1 EML sample was only used to assess for cytokine/chemokine studies. Consistent with their HIV-1 infection status, the group of HIV-infected women had a slightly lower CD4:CD8 T ratio

than the HIV-uninfected women (Figure 1B). T cell activation, however, was similar in HIV-positive and negative women (Figure 1A).

The EML concentrations of several inflammatory molecules, including MCP-1, CXCL-10 and IL-7, were significantly higher in EML than in CVL fluids (Figure 1C–D). In contrast, IL-1α levels were higher in CVL than in EML samples (Figure 1E). These differences were observed regardless of whether we compared samples from the LGT to the UGT of all women or from a single woman (Figure 1E). Markers of monocyte activation, such sCD14 and sCD163, were also elevated in EML compared to CVL samples of HIV-infected and uninfected women (Figure 1C). Several of the increased inflammatory mediators of the UGT were positively correlated with each other. (Figure 1D).

Detection of virus and antiretroviral concentrations

HIV RNA was not detected in matched samples of plasma, CVL and EML (n=8). Proviral DNA was not detected in EMB tissue samples (n=2). This lack of HIV RNA in genital fluids was consistent with antiretroviral drug concentrations in the CVF similar to, or greater than, blood plasma. The active drug concentrations of TFV and FTC (TFVdp and FTCtp) in EMB specimens were greater than concentrations previously documented in the lower genital tract.[34,35] (Table 1).

DISCUSSION

This is the first study to simultaneously assess immunologic, virologic and pharmacologic characteristics of both the UGT and LGT in HIV-1 positive women. These findings supporting the potential for the UGT to exist as a separate compartment for HIV activity are relevant for the prevention and cure of HIV infection.

Since HIV preferentially replicates in activated cells, we studied the cell distribution and state of immune activation of the UGT. Research related to the immunologic effect of HIV on the UGT is limited though the data available shows the endometrium in a chronic inflammatory state in HIV-infected women.[36–38] Enhanced immune activation in endometrial samples of HIV-negative women has previously been reported;[19] this is consistent with our findings that immune activation is high in the endometrium of both HIV-infected and HIV-negative women. These high levels of immune activation imply that there are target cells for HIV transmission, replication and shedding in the endometrium. Increased immune activation has also been described in plasma cells after TFV exposure. [39,40] Our study showed that the immune activation of the endometrium was similar in both HIV-infected women on TDF/FTC and healthy HIV-negative women not taking ARVs in the secretory phase of the menstrual cycle, implying that immune activation of the female UGT represents a normal physiological state.

Genital HIV RNA/DNA shedding has been associated with elevated plasma viral loads and genital tract infection or inflammation.[41–43] Successful suppression of HIV in blood using ART does not eliminate shedding measured in the LGT.[7,44–46] HIV shedding has been assumed to be derived from the mucosal surfaces of the vagina and cervix. However, the endometrium is in direct communication with these LGT sites and it is biologically plausible

for virus to pass from UGT to LGT. A study of untreated HIV-infected women after intrauterine device (IUD) placement found significant increases in genital HIV-1 RNA shedding in women with evidence of chronic endometritis; however, HIV-1 RNA was only measured in the cervix.[38] Ours is the first investigation to measure virus from the LGT and UGT simultaneously and separately in order to localize the exact source of the shedding. Though we did not demonstrate the presence of HIV RNA in the endometrium, our findings were limited by the lower limit of the viral RNA assay of 40 copies/mL and small sample size. It is also possible that because the endometrial lining is routinely shed, there is not enough time within a menstrual cycle to establish a viral reservoir within this compartment.

The observation that suppression of HIV RNA in blood plasma does not always correlate with suppression of virus in the genital tract suggests that the virus may have inadequate ARV exposure in the genital tract. Variable penetration of ARV in different anatomic compartments has been demonstrated in both HIV-infected and healthy women. [29,34,35,47,48] Concentrations of TFV and FTC in CVF in our study were consistent with previously reported concentrations in HIV-infected and healthy females. [34,48] However, our EMB TFV and FTC concentrations were approximately 4-fold higher than vaginal tissue and TFVdp and FTCtp concentrations were 18 and 3-fold higher, respectively.[34] Previous reports of TFVdp in vaginal tissue after oral dosing identify approximately 2% of TFV is converted to TFVdp. [49] In our study, we found a median conversion rate of 18 (17–37)% for TFVdp and 12 (10–13)% for FTCtp. These data suggest that there are different drug distribution influences within the UGT with different intracellular activation kinetics. Though no drug-drug interaction has been demonstrated between TFV and estrogen,[50] an open label study of healthy women reported lower concentrations of serum and intracellular TFV and TFV-dp in those using oral and injectable hormonal contraception.[51]. It is possible that the higher concentrations we noted were due to none of our participants using hormonal contraception; however both the LGT and UGT are equally influenced by sex hormones, and we did not see this difference manifest in the LGT exposure.

To date, the optimal technique to measure HIV shedding in the female genital tract has not been identified. One goal of this study was to develop a sampling and distribution algorithm that would allow the collection of multiple LGT and UGT samples for multidisciplinary analyses. Through unique EML sampling, we were able to obtain cellular and immunologic profiles analogous to those of the endometrium while minimizing trauma and blood contamination associated with EMB samples. Although both sampling methods are effective at studying the female endometrium, the EML may be less invasive and painful due to use of the narrow diameter catheter and lack of suction curettage. Since immunologic studies correlated EML and EMB samples, in the future, we will be able to collect EML samples for immunology while sharing EMB samples between virology and pharmacology labs as needed.

In summary, we found that the endometrium contains activated immune cells. The penetration of TFV and FTC into the endometrium may be at concentrations high enough to prevent viral replication. Though findings from this study do not support the endometrium as a source of genital tract shedding, further research is needed in order to make definitive conclusions on the role of the female UGT in HIV pathogenesis.

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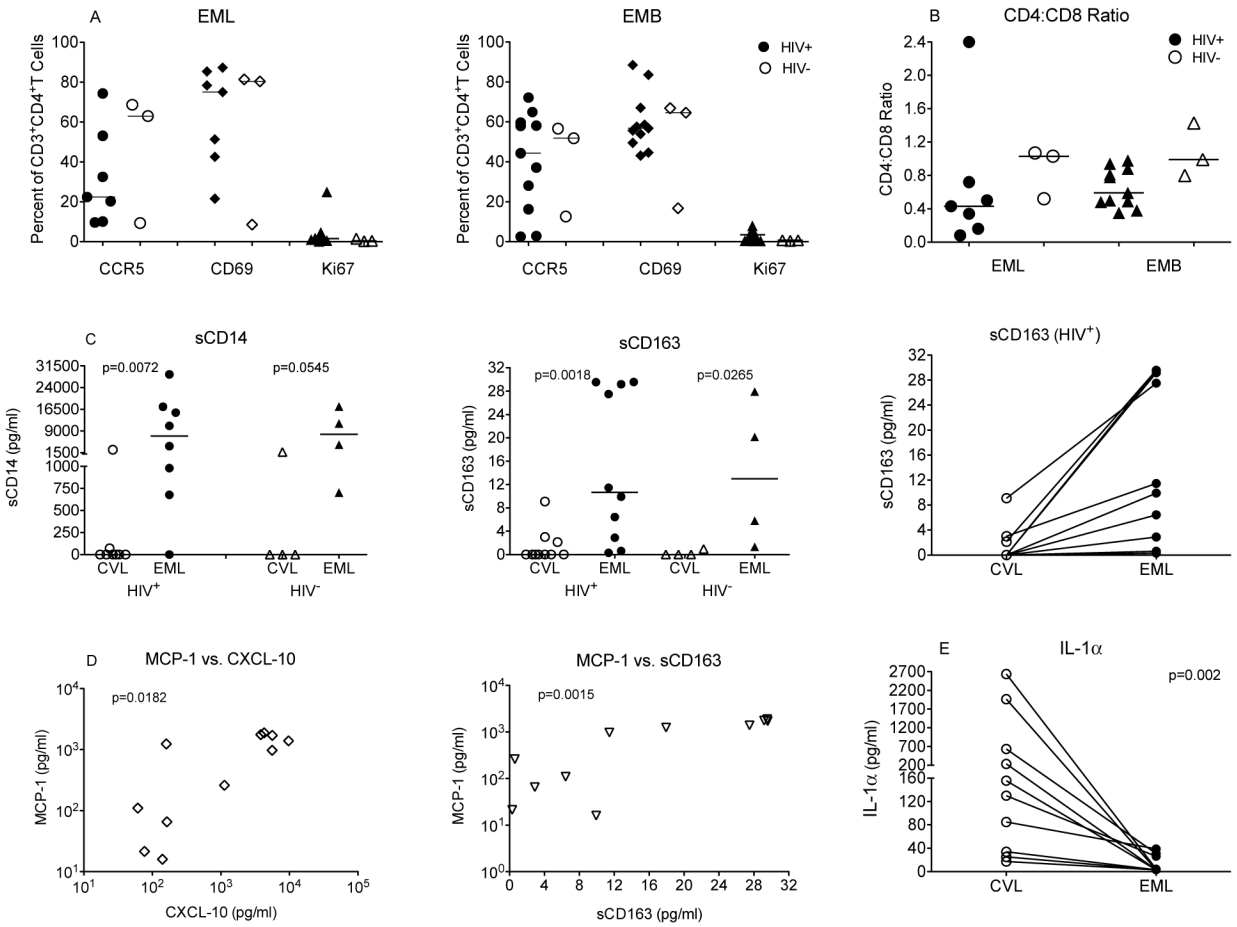

Figure 1. Immune Activation in the upper and lower female genital tract

Panel A: T cell activation in EML and EMB samples of HIV-positive (closed symbols) and HIV-negative (open symbols) individuals as assessed by CCR5, CD69 and Ki67 expression on $CD4^+$T cells. Panel B: Consistent with HIV-1 infection, the CD4:CD8 T cell ratio is reduced in HIV-positive compared to HIV-negative women in EML samples. Panel C: Levels of sCD14 and sCD163 in CVL and EML samples from HIV-positive and –negative women. The right graph illustrates that despite patient-to-patient variation, in each of the HIV-positive women, inflammatory markers (example of sCD163 shown) were expressed at lower levels in CVL compared to EML samples. Panel D: Several of the inflammatory cytokines in EML were positively correlated to each other suggesting that they all serve as surrogate for immune activation. Examples shown include the correlation between MCP-1 and CXCL-10 and MCP-1 and sCD163. Panel E: IL-1α was the only cytokine that was expressed at significantly higher levels in CVL than in EML samples.

Table 1

Nucleoside/tide Reverse Transcriptase Inhibitor (N(t)RTI) [a] and Phosphorylated Metabolite Concentration. Concentrations are reported as median (min, max).

	TFV (nM)	TFV-dp (nM)	FTC (nM)	FTC-tp (nM)
Plasma (N=14)	316 (0.44, 1122) [b]	N/A	977 (155, 9628)	N/A
CVF (N=13)	394 (4, 7869) [c]	N/A	8900 (765, 34021)	N/A
Endometrial Tissue (N=3)	91 (74, 181)	31 (14, 34)	1204 (939, 1617)	161 (115, 163)

[a] The following regimens are represented: TFV/FTC/raltegravir, TFV/FTC/darunavir/ritonavir, TFV/FTC/atazanavir/ritonavir, TFV/FTC/rilpivirine, TFV/FTC/fosamprenavir/ritonavir, and TFV/FTC/elvitegravir/cobicistat.

[b] One sample was below the limit of detection (BLD) and reported as 1/2 the lower limit of detection (LLOQ), 0.25ng/ml.

[c] Two samples were below the limit of detection (BLD) and reported as 1/2 the lower limit of detection (LLOQ), 2ng/ml.