GYNECOLOGY

The effect of menopause on the innate antiviral activity of cervicovaginal lavage

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OBJECTIVE: Reproductive hormones are known to impact innate mucosal immune function of the lower genital tract. Our objectives were to determine the effect of hormonal status on intrinsic antiviral (herpes simplex virus [HSV]-1, HSV-2, and human immunodeficiency virus [HIV]-1) activity of cervicovaginal lavage (CVL).

STUDY DESIGN: CVL was collected from 165 asymptomatic women including postmenopausal women (n = 29); women not on contraception in days 1-14 (n = 26) or days 15-28 (n = 27) of the menstrual cycle; and women using the levonorgestrel intrauterine device (n = 28), depot medroxyprogesterone acetate (n = 28), or combined oral contraceptives (n = 27). The anti-HSV-1/-2 and the anti-HIV-1 activity of the CVL were measured using plaque assays and the Jurkat-Tat-CCR5 assay, respectively.

RESULTS: CVL from all of the groups had modest antiviral activity. Anti-HIV-1 activity was decreased in CVL from postmenopausal women when compared to premenopausal women (11% vs 34%, $P = .002$). However, there was no difference in anti-HIV-1 activity among premenopausal women regardless of phase of menstrual cycle or contraceptive use. Anti-HIV-1 activity was associated with the protein content of the CVL ($r = 0.44$, $P < .001$). There was no difference in anti-HSV-1 or -2 activity by hormonal group.

CONCLUSION: Menopause is associated with decreased innate HIV-1 activity in the lower genital tract, suggesting that factors in the vaginal fluid could play a role in increased susceptibility of HIV-1 infection in postmenopausal women. Hormonal contraceptive use, menopause, and phase of menstrual cycle did not have a measurable impact on the intrinsic anti-HSV-1 or -2 activity.

Key words: contraception, herpes simplex virus, human immunodeficiency virus, innate mucosal immunity, menopause

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The prevalence of human immunodeficiency virus (HIV)-1 infection among older adults is increasing due to improved survival of HIV-infected patients on antiretroviral therapy as well as new primary HIV-1 infections in older patients.[1] In 2009, an estimated 12% of new HIV-1 infections in US women occurred among those >50 years of age.[2] People in this age group are less likely to be knowledgeable about HIV-1,[3] to be screened for HIV-1 or HIV-1 risk factors by their care providers,[4] and to use condoms.[3] Additionally, biochemical, cellular, and structural changes that occur at menopause may increase risk of HIV-1 acquisition in women.[5] Thus, postmenopausal women may be at greater risk for HIV-1 acquisition due to social, behavioral, and biological factors.

Cervicovaginal fluid is composed of cervical mucus, secretions from the upper genital tract, and transudate from the vaginal epithelium and provides an important first-line defense against pathogens, such as HIV-1 and herpes simplex virus (HSV)-1 and -2. Previous studies have shown that cervicovaginal lavage (CVL) has both anti-HIV-1[6-9] and anti-HSV-2[10] activities. Components of the vaginal fluid such as antimicrobial peptides, cytokines, chemokines, and bacterial products contribute to the innate immunity of the female reproductive tract.

The mucosal immune defense of the lower female genital tract has evolved to be responsive to reproductive hormones over the menstrual cycle to balance immune defense to infection with procreation, which is dependent on sperm penetration of the upper genital tract.[11] Keller et al[12] reported that the concentrations of secretory leukocyte protease inhibitor (SLPI), human beta-defensin-2, alpha defensins 1-3, lysozyme, and lactoferrin were lowest at mid-cycle when compared to both the proliferative and secretory stages of the menstrual cycle in CVL samples. However, other studies have shown no effect of menstrual cycle stage on antimicrobial and cytokine levels within female reproductive tract secretions.[10,13,14] These differences could be due to different methods of CVL collection (eg, the amount or type of lavage fluid used or the duration

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of lavage time) or significant intra-individual and interindividual variation.

It is well established that the function of the innate immune system declines with aging[15]; and several studies have demonstrated immune senescence of the lower genital tract mucosal defense.[5] The loss of estrogen after menopause results not only in the thinning of the vaginal epithelium, but also the decline of antimicrobial concentrations in vaginal secretions and protective components of the vaginal microbiome including lactobacilli.[16] With menopause, there is a decrease of cervical mucus production[17] and a significant decrease in vaginal fluid viscosity as measured in CVL.[18] In addition to mediators present in cervicovaginal fluid, changes in the numbers of $CD4^+$ T cells in the genital tract have also been reported in postmenopausal women. Meditz et al[19] compared 22 healthy premenopausal women to 24 postmenopausal women and reported that the percentage of $CD4^+$ T cells expressing the CCR5 receptor in both the cervix and the blood were increased in the postmenopausal women. These authors postulated that postmenopausal women could have an increased risk of HIV-1.

Exogenous reproductive hormones used for contraception impact the concentrations of immune factors in the vaginal fluid, secreted from the epithelial cells lining both the upper and lower genital tract. Secretions from polarized uterine epithelial cells can inhibit growth or kill pathogens, including HIV-1.[20] Incubation of polarized uterine epithelial cells with estradiol increased the capacity of the secretions to inhibit *Staphylococcus aureus* growth, indicating that estradiol modulates expression of immune factors.[21] Endometrial biopsies obtained after exposure to depot medroxyprogesterone acetate (DMPA) showed decreased expression of SLPI compared with biopsies obtained prior to DMPA treatment.[22] The dose, route of administration, and type of progestin may have different effects on the lower genital tract defense. Combined oral contraceptive pills as well as the levonorgestrel intrauterine device have been associated with decreased expression of human beta-defensin-1 and -2, but not SLPI.[23] The primary aim of this study was to characterize the impact of endogenous and exogenous reproductive hormones on the innate anti-HSV-1, anti-HSV-2, and anti-HIV-1 activities of vaginal fluid collected by CVL.

Materials and Methods

Following institutional review board approval by the University of Pittsburgh, informed consent was obtained from healthy, asymptomatic, HIV-negative women who were either between 18-46 years of age or age >50 years. The study population and enrollment procedures were previously described in detail.[18] Briefly, we enrolled women into the study who fell into the 6 following categories: premenopausal women not using exogenous hormones in days 1-14 or in days 15-28 of the menstrual cycle, combined oral contraceptive use, DMPA use, levonorgestrel intrauterine device use, or postmenopausal status based on age >50 years with amenorrhea for at least 1 year. Postmenopausal women receiving hormone replacement therapy were excluded. None of the postmenopausal women reported taking supplements containing phytoestrogens. A vaginal swab for pH, wet mount microscopy, and Gram stain was collected. For collection of the CVL, 10 mL of sterile normal saline was placed into the vagina; a lavage was performed for 1 minute and placed into 15-mL conical vial with 100 μL of protease inhibitor (Sigma-Aldrich, St. Louis, MO), similar to the CVL technique in previous studies of anti-HIV activity.[9] Participant samples were given a unique identifier upon arrival to the laboratory, thus all of the investigators preforming the laboratory assays were masked to the hormonal status of the woman from whom the sample was obtained. All CVL samples were stored at −70°C until they were thawed for immediate testing. The protein content of the CVL samples was determined by the Lowry method.

Anti-HIV-1 assay

The innate anti-HIV-1 activity of CVL was tested using the Jurkat-HIV-1 tat protein-C-C chemokine receptor 5 (Jurkat-Tat-CCR5) assay. Jurkat-Tat-CCR5 cells (kindly provided by Quentin J. Sattentau, University of Oxford) were maintained in Rosewell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum, 2 mmol/L of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin along with 250 μg/mL of hygromycin B (for tat selection) and 500 μg/mL Geneticin (GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT) (for CCR5 selection). After washing, 5×10^4 cells were added to each well of a 96-well plate; each treatment was performed in triplicate. Assays to evaluate the effect of CVL on Jurkat cell viability were performed in parallel with the anti-HIV activity assay. CVL was diluted to 1:16 and added to the appropriate wells and an equal volume of RPMI only (for viability assays) or RPMI containing 3000 median tissue culture infective dose ($TCID_{50}$) of $HIV\text{-}1_{BaL}$ with 2 μg of diethylaminoethanol (for efficacy assays) was added to a final volume of 200 μL resulting in a 1:32 final dilution of the CVL. Control wells for viability assays consisted of medium only and for efficacy assays consisted of medium with HIV-1. The 96-well plates were incubated in a humidified chamber at 37°C/5% carbon dioxide. On day 4, half the volume of culture medium was removed and fresh medium was replenished. On day 7, cell viability and anti-HIV activity was determined. For cell viability, half the medium was removed from the toxicity plate and an equal volume of CellTiter-Glo (Promega, Madison, WI) was added. Luminescence was measured and averaged, and cell viability was determined as deviations from the medium-only wells. For anti-HIV-1 activity, half of the medium was collected and HIV-1 p24gag antigen was quantified by enzyme-linked immunosorbent assay (Alliance; Perkin-Elmer, Waltham, MA) and averaged. HIV-1 suppression was defined as 1 minus the quotient of HIV-1 p24gag antigen with the CVL and the control multiplied by 100. Triplicates rarely differed from each other. When it occurred, the outlier value was removed only if it exceeded 10-fold difference from the other 2 values. Assays were repeated only when the