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Research Article

See Commentaries on p. 18 and p. 24

The Effect of HIV and HPV Coinfection on Cervical COX-2 Expression and Systemic Prostaglandin E_2 Levels

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Abstract

Human immunodeficiency virus (HIV-1) infection causes chronic inflammation. COX-2–derived prostaglandin E_2 (PGE_2) has been linked to both inflammation and carcinogenesis. We hypothesized that HIV-1 could induce COX-2 in cervical tissue and increase systemic PGE_2 levels and that these alterations could play a role in AIDS-related cervical cancer. Levels of cervical COX-2 mRNA and urinary PGE-M, a biomarker of systemic PGE_2 levels, were determined in 17 HIV-negative women with a negative cervical human papilloma virus (HPV) test, 18 HIV-infected women with a negative HPV test, and 13 HIV-infected women with cervical HPV and high-grade squamous intraepithelial lesions on cytology. Cervical COX-2 levels were significantly associated with HIV and HPV status ($P = 0.006$ and 0.002, respectively). Median levels of urinary PGE-M were increased in HIV-infected compared with uninfected women (11.2 vs. 6.8 ng/mg creatinine, $P = 0.02$). Among HIV-infected women, urinary PGE-M levels were positively correlated with plasma HIV-1 RNA levels ($P = 0.003$). Finally, levels of cervical COX-2 correlated with urinary PGE-M levels ($P = 0.005$). This study shows that HIV-1 infection is associated with increased cervical COX-2 and elevated systemic PGE_2 levels. Drugs that inhibit the synthesis of PGE_2 may prove useful in reducing the risk of cervical cancer or systemic inflammation in HIV-infected women. *Cancer Prev Res; 5(1); 34–40. ©2011 AACR.*

Introduction

Human immunodeficiency virus (HIV-1) infection causes chronic inflammation, which is beneficial for HIV-1 replication but detrimental for the human host leading to premature senescence of the immune system, cardiovascular disease, organ fibrosis, and cancer (1–3). The exact mechanisms underlying HIV-induced chronic inflammation and resulting disease are not known.

The inflammatory molecule prostaglandin E_2 (PGE_2) has been linked to carcinogenesis in a number of tumor types including cervical cancer. PGE_2 is secreted from cells and stimulates carcinogenesis by multiple mechanisms (4). PGE_2 promotes angiogenesis (5,6), suppresses apoptosis (7), increases cell proliferation (8), enhances cell invasiveness (9), and suppresses antitumor cell–mediated immunity (10).

The enzyme COX catalyzes the synthesis of prostaglandins from arachidonic acid and is rate limiting in PGE_2 synthesis. COX-2 is overexpressed in transformed cells (11) and in various tumor types (12) including cervical intraepithelial neoplasia (CIN) and cervical cancer (13–15). Notably, human papilloma virus (HPV) oncoproteins E6 and E7 stimulate cervical carcinogenesis, activate *COX-2* transcription, and enhance PGE_2 production (16). Elevated levels of COX-2 correlate with poor prognosis for patients with cervical cancer (17–19). HIV-1 infection induces COX-2 in a number of cell types including circulating monocytes, tissue macrophages, lymphocytes, and neuronal cells (20–25). The up-regulation of COX-2 has been related to several AIDS-defining illnesses including HIV-associated dementia and HIV cardiomyopathy (23–25).

We hypothesized that HIV-1 could upregulate COX-2 in cervical tissue and also increase systemic levels of PGE_2 and that these alterations in prostaglandin metabolism could play a role in HIV-1–related diseases including cervical cancer. Cervical cancer is an AIDS-defining illness, and HIV-1–infected women are 5 times more likely to develop invasive cervical cancer than HIV-negative women (26). We quantified levels of COX-2 in the cervix and systemic PGE_2 levels in HIV-infected versus uninfected women. The study was conducted in Haiti, where cervical cancer is a leading cause of death in HIV-1–infected women (27).

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Methods

Study site

Study participants were recruited at the GHESKIO clinical and research center in Port au Prince, Haiti. GHESKIO provides free HIV voluntary counseling and testing, AIDS care, reproductive health services, and management of sexually transmitted infections. In 2009, the GHESKIO clinic provided HIV voluntary counseling and testing to about 25,000 people and antiretroviral therapy to 6,000 patients with AIDS. Clinical samples were shipped to Weill Cornell Medical College (New York), Columbia University College of Physicians and Surgeons (New York, NY), and Vanderbilt University School of Medicine (Nashville, TN) for laboratory analyses.

Study design

COX-2 mRNA and urinary PGE metabolite (PGE-M) levels, a biomarker of systemic PGE_2, were quantified in 3 groups: (i) HIV-negative women with normal cervical Pap test and a negative test for cervical high-risk HPV DNA (HIV^-/HPV^-); (ii) HIV-1–infected women with a normal cervical Pap test and a negative test for cervical HPV DNA (HIV^+/HPV^-); and (iii) HIV-1–infected women with a high-grade squamous intraepithelial lesion (HSIL) by Pap test and a positive test for cervical HPV DNA (HIV^+/HPV^+). We hypothesized that there would be a stepwise increase in cervical COX-2 mRNA expression in the 3 groups. We also hypothesized that HIV-1–infected women would have higher systemic levels of PGE_2 than HIV-1–negative women. The number of subjects enrolled in each group was determined by feasibility and logistics.

The study was approved by the Institutional Review Boards of the participating institutions. All women in the study provided written informed consent (28).

Patient population

HIV-infected women were recruited from a research cohort examining the effect of early versus deferred antiretroviral therapy (ART; ref. 29). These women were well characterized with CD4 counts and plasma HIV-1 RNA levels measured every 6 months and annual cervical cancer screening with Pap test and HPV testing. Women with a normal cervical Pap test and a negative test for cervical high-risk HPV DNA were recruited. HIV-infected women with high-grade intraepithelial lesions on Pap test and a positive test for cervical high-risk HPV DNA were also recruited. The study visit was scheduled after the screening Pap and HPV test results were available and prior to treatment for the high-grade lesion by cryotherapy or loop electrosurgical excision procedure (LEEP).

HIV-uninfected women were recruited from a cohort of HIV-negative women of reproductive age at high risk for HIV infection followed at GHESKIO. We recruited women with normal cervical Pap test and a negative test for cervical HPV DNA.

Women who converted from HPV negative to HPV positive with low-grade squamous intraepithelial lesions (LSIL) between their annual screening visit and the study visit were not included in the analysis.

Study visit and sample collection

At a single study visit, the Pap test and the HPV test were repeated, urine collected for measurement of PGE-M, and cervical cells collected for quantification of COX-2 mRNA. The study visit was scheduled mid-menses so that cervical samples did not contain menstrual blood. Women with signs or symptoms of a sexually transmitted infection other than HPV were treated and scheduled for their study visit during their next menstrual cycle. Women were asked not to take nonsteroidal anti-inflammatory drugs (NSAID) or aspirin for 2 weeks prior to their study visit. Women taking NSAIDs chronically were excluded.

At the single study visit, women were asked to urinate in a specimen collection container. Urine was aliquoted into three 2-mL cryovials and stored at $-70°C$. A gynecologist collected cervical cells with a cytobrush for HPV and Pap testing in 20-mL Cytyc Thinprep bottles. The gynecologist also collected cervical cells for COX-2 mRNA analysis with a cytobrush by passing it in a complete 360-degree circle around the endocervical junction. The cells were immediately suspended in a cryovial with 2 mL of RNAlater (Qiagen Inc.), placed on dry ice in the clinic, and then transported to a freezer for storage at $-70°C$. The 20-mL Cytyc bottles for Pap test and high-risk HPV testing were shipped to the United States and subjected to analysis as detailed below. The urine and cervical cell suspensions were shipped on dry ice to the United States for analysis.

Laboratory tests

Clinical laboratory tests. The GHESKIO laboratory quantified CD4 T-cell counts by flow cytometry (Becton, Dickinson). HIV-1 viral load was quantified by plasma HIV-1 RNA levels measured by the NucliSens EasyQ HIV-1 PCR Test (BioMérieux).

Liquid-based cytology specimens were routinely processed in the Division of Obstetric, Gynecologic, and Cytologic Pathology at Columbia University Medical Center under the supervision of Dr. T.C. Wright. Results were reported following the Bethesda 2001 classification system as normal, atypical squamous cells of unknown significance (ASC-US), LSIL, or HSIL (30).

High-risk HPV testing was conducted on cervical samples with the Digene Hybrid Capture II HPV DNA Assay (Qiagen Inc.) following manufacturer's instructions. The HC II test is a nucleic acid hybridization assay with signal amplification used to detect and quantify high-risk HPV types in cervical specimens. The test detects 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and is reported as positive or negative for high-risk HPV DNA.

Quantification of COX-2 mRNA

Cervical samples stored in RNAlater were thawed, centrifuged, and the supernatant discarded. Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc.). RNA quantification and quality assessment were conducted using a

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2100 Bioanalyzer (Agilent Technologies). Two hundred nanograms of total RNA was reverse transcribed using murine leukemia virus reverse transcriptase (Roche Applied Science) and oligo(dT)$_{16}$ primer. The resulting cDNA was then used for amplification. Each PCR reaction was 20 µL and contained 5 µL cDNA, 2× SYBR Green PCR master mix, and forward and reverse primers. Primer sequences for amplification were: COX-2, forward: 5′-CCCTTGGGTGTCAAAGGTAA-3′, reverse: 5′-GCCCTCGCTTATGATCTGTC-3′ and β-actin, forward: 5′-AGAAAATCTGGCACCACACC-3′, reverse: 5′-AGAGGCGTACAGGGATAGCA-3′. Experiments were carried out using a 7500 Real Time PCR system (Applied Biosystems). The standard curve method was used to determine levels of COX-2 mRNA using a known copy number of COX-2 mRNA per 10 ng RNA. β-Actin served as an endogenous normalization control.

Urinary PGE-M

Urinary PGE-M is an index of systemic PGE_2 production (31, 32). Catabolism of PGE_2 results in a stable end metabolite, 11α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M), that is excreted in the urine (33–36). PGE-M in urine is stable for prolonged periods of time when stored at −70°C (32). Measurement of urine PGE-M is a better measure of systemic PGE_2 than plasma measurements because PGE_2 in plasma is rapidly metabolized in the lungs and consequently may not accurately reflect endogenous prostaglandin production (37).

PGE-M concentrations in urine were measured in the Eicosanoid Core Laboratory directed by Dr. G.L. Milne at Vanderbilt University. Urine (1 mL) was acidified to pH of 3 with HCl and treated with methyloxime HCl to convert PGE-M to the *O*-methyloxime derivative. The methoximated PGE-M was extracted, applied to a C-18 Sep-Pak, and eluted with ethyl acetate. An [$^2H_6$]-*O*-methyloxime PGE-M deuterated internal standard was then added. Liquid chromatography was conducted using a Waters Acquity UPLC fitted with an Acquity BEH C18 UPLC column (2.0 × 50 mm^2, 1.7 µm) coupled to a Thermo Scientific Quantum Vantage triple quadrupole mass spectrometer. For the subject's urine, the precursor ion of endogenous-formed PGE-M has m/z of 385 and [$^2H_6$]-PGE-M internal standard has m/z of 391 with the expected predominant product ions having m/z of 336 and 339, respectively. Quantification of subject's PGE-M was calculated by ratiometric determinations of unlabeled: labeled peak areas corresponding to both precursor and product ions. The lower limit of detection of PGE-M is in the range of 40 pg, which is approximately 100-fold below levels in normal human urine. Urinary creatinine levels are measured using a test kit from Enzo Life Sciences. The urinary PGE-M levels in each sample are normalized using the urinary creatinine level of the sample and expressed in ng/mg creatinine.

Statistical analyses

Subject characteristics, including age and smoking status, are described for all study participants. For HIV-infected women, ART use, plasma HIV-1 RNA level, and CD4$^+$ T-cell counts at the time of study visit were also summarized and compared among groups defined by HIV serostatus and cervical HPV status. For continuous variables, ANOVA and Kruskal–Wallis methods were used to compare the means and medians, respectively. For categorical variables, Fisher exact test was used to compare the differences in proportions.

Distribution of the 2 primary study endpoints cervical COX-2 mRNA and urinary PGE-M levels in study groups were summarized graphically using box plots. We first used univariate analysis to examine the association between the primary outcome variable and each of the covariates including HIV serostatus, cervical HPV status, age, and smoking status. For the categorical covariates, the association was assessed using the nonparametric Wilcoxon rank-sum test. For age, the association was assessed using univariate linear regression where the outcome variable was log transformed to ensure that the underlying model assumptions were satisfied.

We used multivariate linear regression analysis to adjust for potential confounding and effect modification. A backward variable selection procedure based on the Akaike Information Criteria (AIC) was used to identify the multiple linear regression model that best fit the outcome data (38). The strength of association between each covariate in the best model and the outcome variable was quantified using P values. Similar analyses were then conducted for HIV-positive subjects but with additional covariates, including ART, HIV-1 RNA level, and CD4 counts included in the analysis. P values that are less than 0.05 are considered statistically significant. All the statistical tests are 2-sided.

Results

Study population

Women were recruited between November 2008 and June 2009. Fifty-five women were recruited. Seven women were not included in the study analysis. One HIV-negative and 2 HIV-positive women were excluded because their cervical HPV test converted from negative to positive with LSIL at the time of enrollment. Two HIV-positive/HPV-positive, one HIV-negative/HPV-negative, and one HIV-positive/HPV-negative women had insufficient cervical cells collected for analysis. No one was excluded for chronic aspirin or NSAID use. Complete data are available for 48 women. The characteristics of the 3 groups of study subjects are shown in Table 1. The HIV-negative women were younger than the HIV-positive women; smoking history was similar among groups.

Levels of cervical COX-2 are increased in HIV-infected and HIV/HPV-coinfected women

Cervical cells were collected by cytobrush and stored in RNAlater. Microgram quantities of high-quality RNA were prepared. COX-2 mRNA levels were significantly associated with HIV and HPV status. Distribution of the log COX-2

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Table 1. Characteristics of study population stratified by HIV status and Pap test results

	HIV-negative normal Pap test[a] (*n* = 17)	HIV-positive normal Pap test (*n* = 18)	HIV-positive HSIL on Pap test[b] (*n* = 13)	*P*
Age (mean ± SD)	32 ± 12	43 ± 11	39 ± 8	0.006
Smoking	1 (6%)	1 (6%)	2 (15%)	0.66
Receiving ART	—	15 (83%)	9 (69%)	0.41
Median nadir CD4 T cells per mm^3 (range)[c]	—	228 (31–301)	224 (80–302)	0.89
Median CD4 T cells per mm^3 at time of study (range)	—	402 (181–815)	327 (183–660)	0.07
Median plasma HIV-1 RNA copies per ml at time of study (range)	—	50 (50–240,000)	23,000 (50–630,000)	0.03

[a]Women with normal Pap test had normal cervical cytology by Bethesda Classification system and negative Digene HCII test for high-risk HPV.
[b]Women with HSIL by Bethesda Classification system also had a positive test by Digene HCII for high-risk HPV.
[c]The nadir CD4 T-cell count is the lowest documented CD4 T-cell count for an individual person.

levels stratified by HIV and HPV status are shown with box plots in Fig. 1. Levels of COX-2 mRNA were elevated in the HIV-positive/HPV-negative group compared with the HIV-negative/HPV-negative group ($P < 0.001$). A further increase in COX-2 mRNA levels was observed in the HIV-positive/HPV-positive group compared with the HIV-positive/HPV-negative group ($P < 0.001$). In the HIV-infected women, COX-2 levels were also associated with CD4 counts in multivariate analysis controlling for age. Lower CD4 counts were significantly associated with higher COX-2 expression levels ($P = 0.02$).

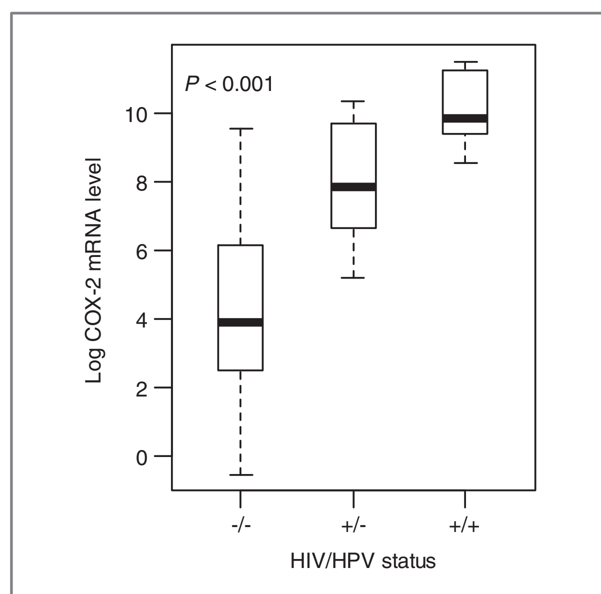

Figure 1. Box plot of the log COX-2 mRNA levels in cervical cells, stratified by HIV and HPV status.

Urinary PGE-M levels are increased in HIV-infected women

The median and range PGE-M values in ng/mg creatinine for the 3 groups were: HIV-negative/HPV-negative group, 6.81 (3.39–22.48); HIV-positive/HPV-negative, 11.05 (5.42–29.86); and HIV-positive/HPV-positive, 12.37 (6.6–24.07). HIV status was the only statistically significant predictor of urinary PGE-M levels in both univariate and multivariate analyses (Fig. 2). HPV infection was not significantly associated with PGE-M levels. Among the HIV-infected women, subjects with higher plasma HIV-1 viral

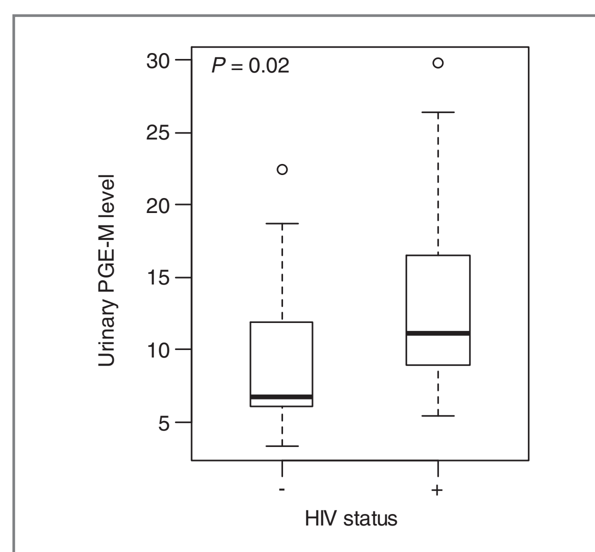

Figure 2. Box plot of urine PGE-M levels in ng/mg in HIV-positive and -negative subjects.

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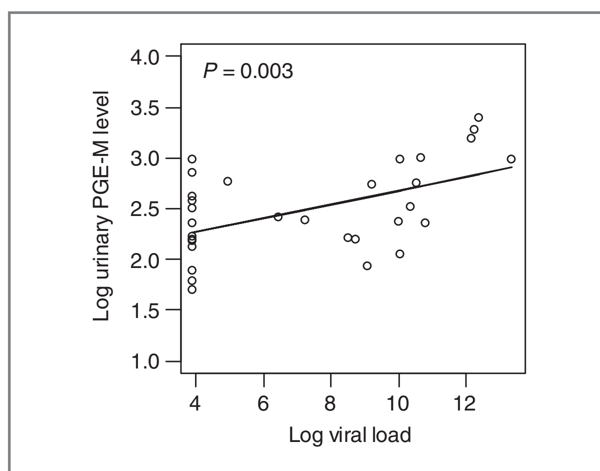

Figure 3. Correlation between log urinary PGE-M levels and log plasma HIV-1 RNA levels in HIV-infected women. We used natural logarithm, so that log(630,000 copies HIV-1 RNA) = 13.35.

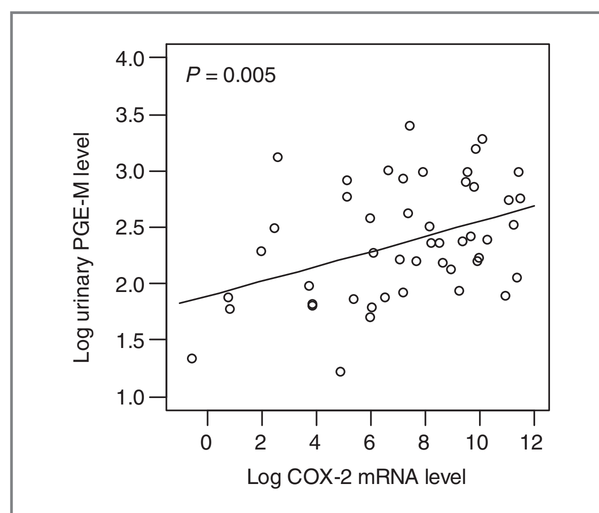

Figure 4. Correlation between log urinary PGE-M levels and log cervical COX-2 mRNA levels.

load had significantly higher urinary PGE-M levels after controlling for age and HPV status ($P = 0.003$; Fig. 3).

Because elevated levels of COX-2 have been associated with increased levels of urinary PGE-M (39, 40), we next correlated levels of cervical COX-2 and urinary PGE-M. Of note, cervical COX-2 mRNA and urine PGE-M levels were positively correlated ($P = 0.005$; Fig. 4).

Discussion

This study validates prior evidence that HIV-1 increases COX-2 expression, although to our knowledge, this has not been previously reported in the cervix. Prior studies have focused upon HIV-1 effects on COX-2–mediated inflammation in neural cells and early-onset dementia. This study extends these findings to cervical cells and systemic PGE_2 levels and suggests a possible link between HIV-1–induced increase in PGE_2 levels and AIDS-related malignancies. The strong correlation between plasma HIV-1 RNA levels and urinary PGE-M levels provides evidence that HIV-1 infection increases systemic production of PGE_2, an immunomodulatory molecule linked to carcinogenesis (10).

Studies have shown that HIV-1 induces COX-2 in neuronal cells, circulating monocytes, tissue macrophages, and lymphocytes (20–23). The upregulation of COX-2 has been implicated in AIDS-related dementia and general immune activation in chronic HIV disease (21–25, 41). We extend these findings to the cervix, where HIV-mediated induction of COX-2 may play a role in cervical carcinogenesis. Notably, COX-2 levels were higher in cervical cells from dual HIV-1/HPV-infected women with squamous intraepithelial neoplasia than cervical cells from women infected with HIV alone. Whether this increase in COX-2 levels reflects cell transformation, a direct effect of HPV E6 and E7 oncoproteins on *COX-2* transcription or both is uncertain (11, 16).

We show that HIV-1 infection is associated with increased levels of urinary PGE-M, which is a measure of systemic PGE_2 levels. This finding is consistent with prior evidence that HIV-1 infection causes chronic inflammation (1–3). In a recent study, increased urinary PGE-M levels correlated with future risk of colorectal cancer (42). In a study of patients with squamous cell head and neck cancer, elevated urinary PGE-M levels correlated with poor prognosis (43). Importantly, in our study, HIV-1 plasma viral load was directly correlated with this potential cancer biomarker. Future studies are warranted to determine whether suppression of HIV-1 replication results in a normalization of urinary PGE-M levels.

HIV-1 infection may increase COX-2 expression and systemic PGE_2 levels through a number of mechanisms. HIV-1 increases COX-2 expression in the T-lymphocytes and macrophages that it infects (20, 21, 23, 25). The HIV-1 transcription factor Tat stimulates COX-2 transcription (44). HIV-1 infection can also increase plasma cytokines including interleukin (IL)-6 and TNFα (45, 46). Each of these proinflammatory cytokines can induce COX-2 and PGE_2 synthesis (12). Destruction of the gut-associated lymphoid tissue by HIV-1 results in translocation of bacteria and elevations of blood lipopolysaccharide (LPS) levels (47). LPS is a potent inducer of COX-2.

Further studies are warranted to elucidate the mechanism (s) by which HIV-1 infection induces COX-2 in cervical cells and enhances systemic PGE_2 synthesis. Although a strong correlation was found between levels of cervical COX-2 and urinary PGE-M, it is highly unlikely that cervical inflammation is responsible for increased systemic PGE_2 levels. It is much more likely that the findings in the cervix reflect a systemic inflammatory process. The correlation between COX-2 expression and systemic PGE_2 levels suggests that elevated PGE_2 results from increased COX-2 activity, likely in systemic lymphocytes and macrophages, but this needs to be verified. Furthermore, prospective studies will need to determine whether elevated COX-2 expression and PGE_2

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levels predict future HPV disease progression and treatment outcomes. This report provides useful biomarkers for these future studies; samples for cervical COX-2 expression and urine PGE-M can be collected noninvasively in settings with high rates of HIV and HPV infection, stored at $-70°C$, and shipped to reference laboratories for analysis.

It is possible that HIV-1–mediated induction of systemic PGE_2 levels contributes to a variety of HIV-related disease processes. For example, COX-2–derived PGE_2 can induce matrix metalloproteinase-9 (48), a proteinase linked to aging-related diseases including coronary artery disease, cancer, and early emphysema in HIV-infected individuals (49). Studies are warranted to determine whether urinary PGE-M levels predict future occurrence of diseases related to chronic inflammation in HIV-1–infected people (1–3).

This study enrolled relatively small numbers of women and did not enroll women with HPV and low-grade dysplasia. Therefore, we do not know the independent effect of HPV infection versus cervical cell transformation on COX-2 levels. Larger studies are needed to validate our findings and to examine cervical COX-2 in women with HPV infection and different grades of dysplasia.

This study shows that HIV-1 infection is associated with increased amounts of cervical COX-2 and elevated systemic PGE_2 levels. Drugs that inhibit the synthesis of PGE_2, including aspirin, selective COX-2 inhibitors, and traditional NSAIDs, may prove useful in reducing the risk of cervical cancer and systemic inflammation in HIV-infected women.

Disclosure of Potential Conflicts of Interest

A.J. Dannenberg is a member of the Scientific Advisory Board of Tragara Pharmaceuticals, Inc., a company that is developing a selective COX-2 inhibitor. D.W. Fitzgerald supervises a scholarship program for Tanzanian medical students sponsored by Pfizer Inc. No potential conflicts of interest were disclosed by other authors.

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Cancer Prevention Research

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