Infect Dis Obstet Gynecol 2002;10:181–186

Role of the vaginal microbiological ecosystem and cytokine profile in the promotion of cervical dysplasia: a case–control study

Kian Behbakht[1], Jennifer Friedman[1], Ira Heimler[1], Alla Aroutcheva[1], Jose Simoes[1] and Sebastian Faro[2]

[1]*Department of Obstetrics and Gynecology, Rush Medical College, Chicago, IL*
[2]*The Woman's Hospital of Texas, Houston, TX*

Objective: To identify alterations in the cytokine profile and microbial ecosystem of the vagina in association with cervical dysplasia.

Methods: Demographics, lifestyle variables and Papanicolau (Pap) smear results of subjects presenting to the same site for gynecologic complaints, obstetric visits or colposcopy were prospectively recorded. Vaginal smear for Gram stain, aerobic and anaerobic culture, pH, and wet mount and KOH examination for *Trichomonas vaginalis*, *Gardnerella vaginalis* and yeast organisms were performed. Vaginal lavage specimens were centrifuged, and the pellets and supernatants were assayed for human papillomavirus (HPV) by polymerase chain reaction and for cytokines interleukin (IL)-1β IL-6, IL-10 and IL-12 by enzyme-linked immunosorbent assay (ELISA) respectively. Subjects with abnormal Pap smears underwent colposcopy and biopsy as indicated.

Results: Of 51 patients, 32 were referred for colposcopy, 12 presented with gynecologic needs, and seven presented for obstetric visits. Median age was 24 years. Demographics did not differ significantly between the dysplasia and control groups except for a trend towards more sexual partners in the dysplasia group. Biopsies were performed in 81% (26/32) of patients presenting for colposcopy and 17 revealed cervical intraepithelial neoplasia. IL-1β, IL-6, IL-10, and IL-12 levels were elevated in 63% (20/32), 38% (15/39), 4% (2/49), and 0% of samples respectively. Elevated vaginal lavage IL-1β was associated with a 6.1 odds ratio (95% confidence interval 1.06–35) of cervical dysplasia. Alterations in other variables studied were not associated with cervical dysplasia.

Conclusions: Elevated IL-1β, possibly representing a complex host inflammatory response to multiple pathogens, was demonstrated in patients with cervical dysplasia.

Key words: CERVICAL NEOPLASIA, INTERLEUKINS, BACTERIAL VAGINOSIS

INTRODUCTION

Neoplasms of the uterine cervix are a public health problem. In the United States, women affected annually include 2.5 million with cervical dysplasia, 50 000 with cervical carcinoma *in situ* and 16 000 with cervical carcinoma[1,2]. While early detection may decrease advanced disease, altering exposure will reduce the occurrence of disease and is a preferred strategy.

Some studies show a higher incidence of dysplasia in women with bacterial vaginosis (BV)[3,4]. Alternatively, the presence of inflammatory cytokines related to alterations in the vaginal

Correspondence to: Kian Behbakht, MD, 4200 East 9th Avenue, Box B-198, Denver, CO 80209, USA.
Email: kian.behbakht@uchsc.edu

environment, and not the presence of the pathogens themselves, might play a role as a promoting factor[5]. In support of this, increased levels of IL-1β were seen in the cervical mucus of pregnant patients with BV. Furthermore, cervical mucus from these women with BV induced production of IL-6 from monocytic cells *in vitro*[6]. In another study, a shift to TH2 cytokines (IL-4, IL-10) was observed with increasing grades of cervical dysplasia[7]. Therefore, changes in the microbial flora, cytokine profile, or both may predispose to cervical dysplasia.

As an alternative to cervical mucus assays, cytokine concentrations in the vaginal environment can be evaluated using vaginal lavage. Using this technique, Sha and co-workers demonstrated increased IL-1β levels in HIV-infected patients with cervical dysplasia compared with HIV-infected patients without cervical dyspalsia[8]. In this study, we prospectively evaluated the clinical presentation, presence of infection, and cytokine profile, via vaginal lavage, of patients with and without cervical dysplasia.

MATERIALS AND METHODS

The study population included all patients aged 13–65 years presenting to outpatient facilities at Rush-Presbyterian-St. Luke's Medical Center between January and October 2000. Participants signed an Institutional Review Board (IRB)-approved consent specifically for this study. Patients included those with abnormal Pap smears presenting for colposcopy to the study authors and additional patients presenting to the same site for routine gynecologic or obstetric care.

The sample group consisted of patients with biopsy-proven dysplasia, and the control group consisted of those with normal Pap smear, normal biopsies or a colposcopic examination that ruled out a high-grade lesion.

Initial visits consisted of study explanation, signing of the consent form and addressing the reason for the visit. Information was then collected including: age, parity, age at first intercourse, smoking history, history of sexually transmitted diseases, and number of sexual partners. Cervical and vaginal specimens for vaginal pH, wet mount for the presence of *Trichomonas vaginalis*, KOH prep for the presence of yeast, Gram stain with evaluation of altered vaginal flora using Nugent's criteria[9], and aerobic and anaerobic cultures were obtained. Subsequently, the cervix and vagina were lavaged with 5 cm^3 of sterile saline and specimens centrifuged to yield supernatants for cytokine profile assays and pellets for human papillomavirus (HPV) determination. Interleukin (IL)-1β, IL-6, IL-10, and IL-12 levels were assayed by enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, MN). HPV was assayed by polymerase chain reaction (PCR) using L1 consensus primers (5′-TTT GTT ACT GTG GTA GAT AC-3′, 5′-GAA AAA TAA ACT GTA AAT CA-3′; annealing temperature, 45°C).

Lactobacilli from vaginal specimens were isolated by inoculating MRS agar (Remel, Lenexa, KS), incubated anaerobically at 36°C for 24–48 hours. *Gardnerella vaginalis* was isolated by inoculating the vaginal specimens on human-blood-bilayer tween (HBT) agar (Becton Dickinson, Baltimore, MA) and incubating in 5% CO_2 atmosphere at 37°C for 48 hours. Sabouraud agar (Hardy Diagnostics, Santa Monica, CA) was used for isolation of yeast. *Trichomonas vaginalis* was isolated by inoculating the vaginal specimens on Diamond media (Queleb, Montreal, Canada) and incubating at 37°C for 7 days.

All of the wet mount, KOH prep and vaginal pH assessments were analyzed by one of two investigators using standard methodology[10]. One investigator performed all the colposcopies and biopsies. Pap smear reports were reviewed, but slides were not reviewed again by a cytopathologist specifically for this study.

SPSS Version 11 for Windows (SPSS, Chicago, IL) was used for data management and statistical analysis. Groups were compared with respect to categorical variables using the chi-square test of association or Fisher's exact test, and with respect to non-categorical, statistically non-normal variables using the Mann–Whitney U-test. A 0.05 significance level was used for all statistical tests.

RESULTS

Of 51 patients included in the study, 32 were referred to a colposcopy clinic, 12 presented with

gynecological needs, and seven presented for obstetric visits. Overall, 18 pregnant women were admitted to the study including those presenting for colposcopy. Approximately 78% (40) and 15.7% (eight) of the patients were African-American and Hispanic respectively. Seventeen patients (33%) with biopsy-proven dysplasia were the sample patients; 34 (67%) with normal biopsies or a colposcopic exam that ruled out dysplasia were the control patients.

Study groups did not differ significantly in continuous variables (Table 1) such as age, age at first intercourse, tobacco use and parity. There was a trend towards more sexual partners in the dysplasia group ($p = 0.08$ by chi-square). Groups also did not differ significantly in categorical variables (Table 2) such as history of sexually transmitted disease (STD), presence of BV by clinical criteria[11] or Gram stain or culture, presence of HPV, or elevated vaginal fluid IL-6 (greater than 2 pg/ml). Vaginal IL-1β concentration was significantly elevated in 11/13 (85%) of dysplasia patients compared with 9/19 (47%) of controls ($p = 0.03$ by chi-square). Odds ratio (OR) for cervical dysplasia was 6.1 (95% confidence interval (CI) 1.06–35) in patients with elevated IL-1β. Mean IL-1β was 45 pg/ml (range 0–125 pg/ml) in the no dysplasia group and 89 pg/ml (range 8–358 pg/ml) in the dysplasia group. Elevated vaginal IL-1β was correlated with a higher number of previous sexual partners (mean of two partners for normal vaginal IL-1β vs. mean of seven for elevated vaginal IL-1β, $p = 0.009$, Mann–Whitney U-test). Elevated IL-1β was not correlated with the presence of BV by Gram stain. IL-12 was not elevated in any specimen and IL-10 was present in two specimens, one contaminated with menstrual blood.

Four of the patients studied had data collected on two separate visits. Of interest, two of these patients had elevated IL-6 found on the return visit only. One patient had a biopsy-proven herpetic outbreak on the follow-up visit alone (IL-6 = 300 pg/ml), and the other was menstruating at the follow-up visit (IL-6 = 75 pg/ml).

Finally, HPV was detected in 3/9 (33%) patients with biopsy-proven high-grade lesions compared

Table 1 Comparison of continuous variables between dysplasia and no dysplasia groups

Variable	*Dysplasia group, mean ± standard deviation (n = 17)*	*No dysplasia group, mean ± standard deviation (n = 34)*	*Significance*
Age	25 ± 7	24 ± 10	NS
Age at first intercourse	16 ± 2	16 ± 3	NS
Life-time number of sexual partners	7 ± 7	4 ± 3	NS
Pack-years' cigarette smoking	1.6 ± 2.5	1.1 ± 3.3	NS
Parity	2 ± 2	2.5 ± 2.5	NS

NS, not significant

Table 2 Comparison of categorical variables between dysplasia and no dysplasia groups

	Dysplasia group	*No dysplasia group*	*Statistical significance*
History of STD	9/17 (53%)	22/34 (65%)	NS
Clinical BV	1/17 (6%)	5/34 (14%)	NS
Gram stain BV	10/17 (59%)	15/34 (44%)	NS
Presence of HPV	3/17 (17%)	2/34 (6%)	NS
Elevated IL-6 concentration > 2 pg/ml*	8/15 (53%)	7/24 (29%)	NS
Elevated IL-1β concentration > 2 pg/ml**	11/13 (85%)	9/19 (47%)	$p = 0.033$

*Data were not available for all patients. Numbers exclude two patients with extremely high values, one with active HSV infection and another with specimen contaminated with menstrual blood; **data were not available for all patients. Numbers exclude two patients with extremely high values, both contaminated with menstrual blood; STD, any episode of pelvic inflammatory disease, chlamydia, or Gonorrhea cervicitis; BV, bacterial vaginosis; HPV, Human Papillomavirus; NS, not significant

with 2/32 (6%) patients with low-grade lesions or no cervical dysplasia ($p = 0.06$ by Fisher's exact test). All specimens testing positive for HPV also demonstrated elevated IL-1β (mean 35 pg/ml, range 10–60 pg/ml).

DISCUSSION

The presence of HPV in human cervical cells has been shown to be an initiating factor in the development of cervical dysplasia[12]. However, not all patients harboring HPV develop cervical dysplasia, indicating the presence of additional promoting factors. Multiple studies have shown that patients with BV have a higher incidence of cervical dysplasia[3,4,13]. In one study, the production of nitrosamines by the causative organisms of BV was shown to promote the development of cervical dysplasia and cervical cancer[5]. However, the interactions in the vaginal mircoenvironment are complex and higher incidence may not be related to the pathogens themselves, but to alterations in the microenvironment.

Bacterial vaginosis is a polymicrobial syndrome involving the lower genital tract and is characterized by the replacement of lactobacilli-predominant flora with *G. vaginalis*, anaerobes and *Mycoplasma hominis*. Furthermore, some biotypes of *G. vaginalis* are more strongly associated with clinical BV[14]. This polymicrobial infection results in alterations in the vaginal microenvironment leading in the extreme to clinical presentation of vaginal itching, a malodorous discharge, a positive amine 'whiff' test and the presence of clue cells on a wet smear. However, shifts in vaginal flora are detectable on Gram stain prior to the onset of clinical symptoms. It is therefore possible that silent infection may be present in association with other diseases.

Matzby-Baltzer and co-workers found elevated levels of IL-1β in the lower genital tract of pregnant women with asymptomatic BV[6]. Furthermore, cervical mucus from patients with BV-induced IL-6 secretion from monocytic cells *in vitro*. Sjoberg and co-workers showed that BV-associated endotoxin is responsible for the production of lipopolysaccharides capable of inducing an inflammatory response[15]. In our study, elevated IL-1β or IL-6 was not associated with BV. However, elevated IL-1β was associated with a higher number of previous sexual partners, itself a risk factor for bacterial vaginal infections, infection with HPV, and cervical dysplasia. It is possible that endotoxin production is related to the biotype of *G. vaginalis* and its interaction with other pathogens in the vaginal environment. We do not have biotype data for our *G. vaginalis* isolates.

In our study, elevated IL-1β was associated with the presence of cervical dysplasia. This is similar to findings of elevated vaginal lavage IL-1β in HIV-positive patients with cervical dysplasia[8] and is supported by an association between cervical dysplasia and a greater percentage of cells genetically programmed to secrete IL-1β in cervical biopsies[16]. IL-1β has been shown to stimulate HIV replication *in vitro* by activation of the long terminal repeat promoter region[17]. We speculate that increased IL-1β may similarly promote HPV replication. This is supported by our finding by PCR of elevated IL-1β in patients with HPV infection. However, we have no direct evidence of this relationship in our study.

IL-10 is a pleiotropic cytokine that is thought to have immunosuppressive effects on the cervical epithelium. It is not clear why IL-10 was only elevated in one of our specimens. Mota and co-workers demonstrated increased IL-10 expression with progression from normal cervical epithelium, then low-grade, to high-grade lesions[18,19]. Their data, however, were based on immunohistochemical detection of IL-10 in biopsy specimens, not from vaginal lavage. It is possible that IL-10 may not be detectable in vaginal lavage while elevated at the tissue level. In relation to changes in the local environment, presence of the cytokine in vaginal lavage fluid may be more important.

IL-6 is also a multifunctional cytokine, but is thought to promote an inflammatory response in the cervical epithelium. Levels of vaginal lavage IL-6 were < 2 pg/ml in controls, < 2–73 pg/ml, mean 22 pg/ml in patients with cervical dysplasia and 54–780 pg/ml, mean 171 pg/ml in patients with cervical cancer in one study[20]. Our study did not include patients with cervical cancer. Although the mean IL-6 level in our patients with dysplasia (18 pg/ml, range 0–75 pg/ml) was

higher than in controls (11 pg/ml, range 0–70 pg/ml), this difference was not significant.

Biases in our study population require discussion. The predominance of African-American and Hispanic patients in our study reflects demographics of an urban practice. These results may not be applicable to patients in alternate demographic settings. The median age of 22 years in our study and control population was below that expected for cervical dysplasia and it is conceivable that some of these women are at high risk for future development of cervical dysplasia. It is possible that continued exposure, not a one-time event, might be the risk factor for eventual development of dysplasia. For example, patients with elevated IL-1β without cervical dysplasia may be at risk for development of cervical dysplasia in the future.

A disadvantage of the vaginal lavage procedure is that dilution of cervical secretions is not known with absolute confidence. While data exist on conversions from volume-standardized vaginal lavage levels to cervical mucus levels[21], we chose to report our results as the categorical variable (elevated or not elevated) rather than the continuous variable (cytokine level). We report the cytokine levels for reference to existing literature.

We also recognize the 5/39 (13%) prevalence of HPV in our study is below that expected for this population. This may be related to our method of assaying HPV from cells shed in the vaginal lavage. It is possible that one-time assay of HPV may miss many patients that carry HPV but are not actively shedding virus. However, all patients demonstrating HPV virus in vaginal lavage specimens also exhibited elevated IL-1β, suggesting a relationship between the presence of virus in the vaginal environment and the presence of this cytokine.

Elevated IL-1β is associated with cervical dysplasia, but not with Gram stain presence of BV. Presence of HPV in vaginal fluid may be related to secretion of IL-1β. The cause of elevated IL-1β is unclear but may be related to multiple factors resulting in an altered vaginal microenvironment. Identification of these factors and their alleviation may help reduce the incidence of cervical dysplasia.

REFERENCES

1. Kurman RJ, Henson DE, Herbst AL, *et al*. Interim guidelines for management of abnormal cervical cytology. The 1992 National Cancer Institute Workshop. *J Am Med Assoc* 1994;271:1866–9
2. Wingo PA, Tong T, Bolden S. Cancer Statistics, 1995. *CA Cancer J Clin* 1995;45:127–8
3. Boyle DCM, Uthayakumar S, Hlaket J, *et al*. Does bacterial vaginosis cause cervical neoplasia [abstract]. Proceedings of the 29th Annual Meeting of the Society of Gynecologic Oncologists, February 7–11, 1998;108
4. Guijon F, Paraskevas M, Rand F, *et al*. Vaginal microbial flora as a cofactor in the pathogenesis of uterine cervical intraepithelial neoplasia. *Int J Gynecol Obstet* 1992;37:185–91
5. Pavic N. Is there a local production of nitrosamines by the vaginal microflora in anaerobic vaginosis/trichomoniasis? *Med Hypotheses* 1984;15:433
6. Mattsby-Baltzer I, Platz-Christensen JJ, Hosseini N, Rosen P. IL-1B, IL-6, TNFa, fetal fibronectin, and endotoxin in the lower genital tract of pregnant women with bacterial vaginosis. *Acta Obstet Gynecol Scand* 1998;77:701–6
7. Clerici M, Merola M, Ferrario E, *et al*. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. *J Natl Cancer Inst* 1997;89:245–50
8. Sha BE, D'Amico RD, Landay AL, *et al*. Evaluation of immunologic markers in cervicovaginal fluid of HIV-infected and uninfected women: implications for the immunologic response to HIV in the female genital tract. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997;16:161–8
9. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standarized method of Gram stain interpretation. *J Clin Microbiol* 1991;29:297–301
10. Amsel R, Totten PA, Spiegel CA, *et al*. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. *Am J Med* 1983; 74:14–22
11. Sonnex C. The amine test: a simple, rapid, inexpensive method for diagnosing bacterial vaginosis. *Br J Obstet Gynecol* 1995;102:160–1
12. Bedell MA, Jones KH, Laimins LA. The E6–E7 region of human papillomavirus type 18 is

sufficient for transformation of NIH 3t3 and Rat-1 cells. *J Virol* 1987;61:3635–40
13. Platz-Christensen JJ, Sundstrom E, Larsson P. Bacterial vaginosis and cervical intraepithelial neoplasia. *Acta Obstet Gynecol Scand* 1994;73:586–8
14. Aroutcheva AA, Simoes JA, Behbakht K, Faro S. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin Infectious Dis* 2001;33: 1022–7
15. Sjoberg I, Hakansson S. Endotoxin in vaginal fluid of women with bacterial vaginosis. *Obstet Gynecol* 1991;77:265–6
16. Majeed GS, Glew S, Bidwell J. An association between LSIL and the high secretor phenotype of IL-1β. *Gynecol Oncol* 1999;73:359–61
17. Strum-Ramirez K, Gaye-Diallo A, Eisen J, *et al.* High levels of tumor necrosis factor-α and interleukin-1β in bacterial vaginosis may increase susceptibility to human immunodeficiency virus. *J Infect Dis* 2000:182:467–73
18. Mota F, Rayment N, Chong S. The antigen-presenting environment in normal and human papillomavirus-related premalignant cervical epithelium. *Clin Exp Immunol* 1999;116:33–40
19. Mota F, Calder V, Rutault K, *et al.* Characterization of soluble factors from cultures of premalignant cervical epithelium. *Eur J Gynaecol Oncol* 1999;20:262–7
20. Tjiong M, Van der Vange N, Kate F, *et al.* Increased IL-6 and IL-8 levels in cervicovaginal secretions of patients with cervical cancer. *Gynecologic Oncology* 1999;73:285–91
21. Belec L, Meillet D, Levy M, *et al.* Dilution assessment of cervicovaginal secretions obtained by vaginal washing for immunological assays. *Clin Diagn Lab Immunol* 1996;104:538–42

RECEIVED 03/27/02; ACCEPTED 06/14/02