RESEARCH COMMUNICATION

Serum IL-6 and Micrometry of Pap Smears in Women with Cervical Low-Grade Intraepithelial Lesions

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Abstract

Objective: To assess serum IL-6 in women with or without low-grade squamous intraepithelial lesions (LSILs) in Pap smears and correlate with the nucleo/cytoplasmic (N/C) ratio. Methods: Manual micrometry was carried out on Pap smears for N/C ratios: Group A, negative findings (N=15); Group B, inflammation without abnormality (N=14); Group C, LSIL with inflammation (N=13). Serum IL-6 was measured in Groups B and C after treatment of nonviral genital infections. Women with pelvic inflammatory or systemic diseases were excluded. Results: The N/C ratio was significantly higher in Group C vs Group B , both before and after treatment of nonviral infections and also vs group A ($p < 0.001$, Students t test). After treatment for non-viral infections serum IL-6 levels were >50pg/ml in 5/13 cases of Group C and significantly higher than levels in Group B ($p<0.05$), correlating positively with the N/C ratio in the 13 cases of LSIL (Pearson's coefficient $r=0.659$, $p<0.05$). Conclusions: High peripheral circulating level of IL-6, despite prior treatment of nonviral infections, was observed in more than one third of women with persistent LSIL in Pap smears, and may serve as an additional biomarker for early cervical neoplasia. Long term follow up is indicated.

Keywords: Pap smears - IL-6 - micrometry - LSIL - cervical cancer

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Introduction

Chronic inflammation, in particular infection with high risk human papilloma virus (HPV) types is known to be the major culprit in cervical carcinogenesis (Zur Hausen, 2009) along with other risk factors (Joshi et al., 1993; Gariglio et al., 2009; Jayshree et al., 2009; Kwasniewska et al., 2009). Routine screening with Papanicolaou (Pap) smears has demonstrated that cervical carcinogenesis is a multistep process, thus allowing precancerous changes to be detected (Bethesda System, 2001; Weinberg, 2007). Precancer or cervical intraepithelial neoplasia (CIN), corresponding to low-grade squamous intraepithelial lesion (LSIL) in Pap smears occurs several times more often than cancer, and is reversible in 40 to 90% of cases; specially in teenage population (Moscicki et al., 2004; Weinberg, 2007). Since logistically it is difficult to follow up several thousands of women regularly for decades, several attempts have been made to find biomarkers which will identify women at high risk (Janicek et al., 2001; Adamopoulou et al., 2009).

Interleukin-6 (IL-6) is a proinflammatory pleotrophic cytokine associated with various types of infections and cancers (Tjiong et al., 1999; Salgado et al., 2002; Pardo-Govea et al., 2005; Heikkila et al., 2008). IL-6 values in vaginal fluid are increased in cervical cancer and precancer, including LSIL. Circulating levels of IL-6 are also reported to be high in gynaecological cancer.

We have observed persistence of LSIL even after treatment of local nonviral infections (Joshi et al., 2010). We have also observed raised serum IL-6 levels in women with abnormal Pap smears as compared to women with inflammatory smears (Unpublished data). Hence it was of interest to determine serum IL-6 levels in women with persistent LSIL after treatment of infections and compare them with serum IL-6 levels in women with inflammatory smears.

Micrometric changes reflect precancerous grades in cervical neoplasia (Foraker and Reagan, 1965; Bollmann et al., 2001; Steinman et al., 2008), therefore we also wanted to correlate the serum IL-6 levels with micrometric changes in women with LSIL. Moreover circulating level of IL-6 has shown correlation with histological stage of cervical neoplasia in experimentally induced cervical cancer in the rat model (Bustamam et al., 2008). We have earlier reported the effect of treatment of nonviral genital infections on cytologic patterns and specific infections (Joshi et al., 2004; Joshi et al., 2010; Paradkar et al., 2010) but have not reported on the micrometric changes. We have also not come across published data on micrometry and serum IL-6 levels in women with inflammatory Pap smears, before and after treatment of infections.

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This study is part of baseline data on effect of various treatments on clinical symptoms and cytology and was approved by an independent Ethics Committee. The study was observational, based on rescreening of Pap smears and serum IL-6 measurements in selected subjects with inclusion / exclusion criteria from a total of 1067 women participating in a screening program over a 4 year period. All women with higher grades of abnormality were referred for appropriate hospital management.

Objective

1) To determine micrometric changes in Pap smears with inflammation alone vs inflammation with LSIL, before and after treatment of nonviral infections, and compare them with Negative smears .

2) To study serum IL-6 levels in women with inflammatory smears before and after therapy for nonviral infections, and compare them with levels in women with a Pap smear report of persistent LSIL.

Subjects & methods: In order to avoid confounding factors only those cases which satisfied the following inclusion and exclusion criteria were included in this analysis:

Inclusion criteria

1) Women who were willing for treatment as per national guidelines for treatment of RTIs / STDs 2) Tubal ligation or consistent condom use for contraception 3) Abstinence during treatment period 4) Women willing for basic clinical biochemistry 5) Informed written consent

Exclusion criteria

1) Pelvic Inflammatory Disease 2) Pap smear with High Grade Intraepithelial Lesion or Cancer 3) Unexplained bleeding 4) Pregnancy 5) Fibromyoma, polyp, ovarian tumors, prolapse 6) Uncontrolled systemic diseases like hypertension, diabetes 7) Hemoglobin < 9 gms % 8) Positive VDRL or HIV test.

Treatment given

Selected women underwent standard treatment with antifungal, antibacterial and antiprotozoal therapy as reported earlier as per national guidelines alongwith their sexual partners (Joshi et al., 2010). The following drugs were used: Azithromycin, Secnidazole or Satronidazole, Fluconazole, and Clotrimazole. Wo men with persistent discharge / erosion after 2 weeks were treated with Cephadroxyl for presumptive gonorrhea.

Collection and Evaluation of Pap smears

Ectocervical and endocervical smears were collected with a disposable spatula and brush (Cytobrush ® Plus GT, Medscand Medical), spray-fixed, stained with Papanicolaou stain and interpreted as per the Bethesda System (Bethesda 2001, Mali et al., 2004).

After screening the women were classified into the following 3 groups:

Group A - Negative (N=15) : For micrometry the controls were derived from women with negative smears from the same program. These women did not require treatment and in this group only routine clinical biochemistry was studied.

Group B - Inflammation (N=14) : Pap smear report was inflammation only, no abnormal cells seen. In this group micrometry and serum IL-6 measurements were carried out before and after treatment of nonviral infections.

Group C - LSIL (N=13) : Pap smear report was LSIL with inflammation , initially and persistent LSIL after treatment of nonviral infections. In this group micrometry was carried out before and after treatment of infections; serum IL-6 was measured after treatment of infections.

Manual micrometry was carried out on Pap smears (Oil immersion X 100) for nuclear and cell diameters (Foraker and Reagan,1965; Bollmann et al, 2001; Steinman et al, 2008). Cells from five most abnormal fields were studied.

All women in Groups A, B and C had complete blood count , erythrocyte sedimentation rate, fasting blood sugar, serum cholesterol, serum liver enzymes, and serum creatinine, VDRL and HIV testing in blood samples as part of routine biochemical testing to exclude major systemic diseases.

Serum IL-6 : This was measured by ELISA in Group B and C, using a commercial kit (Biosourse IL-6 EASIA kit; KAP 1261). The controls were derived from 10 asymptomatic clinically healthy young males and females (18-35 years).

Results

The mean (± SEM) age , weight, Body Mass Index (BMI) and parity were 33.1±2.0 years, 52.3±3.2 Kg , 22.16±1.26 and 1.73±0.24 in Group B, and 40.5±2.7 years, 57.1±2.7 Kg, 23.66±1.37 and 1.67±0.25 in Group C.

Compliance to treatment and follow up was good in all women and their partners included in study. Only 3 patients reported mild gastrointestinal side effects. Routine

Table 1. Mean (SEM) Nuclear Diameters and N/C Ratio in Pap Smears from Group A, Groups B and C, Before and After Treatment of Infections

Micrometry		Group A N=15	Group B N=14		Group C N=13	
			Before	After	Before	After
Nuclear diameter*(μ)	Mean	10.66	11.51	11.14	13.56	13.61
	SEM	0.25	0.38	0.24	0.48	0.46
N/C ratio**	Mean	0.26	0.33	0.31	0.35	0.36
	SEM	0.009	0.02	0.02	0.01	0.02

* p< 0.001 Group C vs Group A; ** p < 0.001 Group C vs Group A and p<0.05 Group C vs B , Student t test

biochemistry was within normal limits in all 3 groups.

Pap smear analysis

a) Cytological manifestations of infections: Pap smears showed an improved smear pattern after treatment in both groups but few, in both groups, turned negative. Cytological manifestations suggestive of Fungal hyphae, Trichomonas, Chlamydiasis and Bacterial vaginitis were reduced. Koilocytosis, suggesting Human Papilloma Virus (HPV) infection, and few clue cells suggestive of Bacterial vaginosis persisted in both groups (Joshi et al., 2010).

b) Micrometry: Micrometric findings in Groups A, Group B (Before and After treatment) and Group C (Before and after treatment) are given in Table 1.

Micrometric measurement differed significantly between the groups but did not show significant changes in response to treatment in both groups B and C. The mean nuclear diameter was significantly more in Group C vs Group A ($p<0.001$ Student t test). The N/C ratio in LSIL Group C was significantly higher vs Group B and Group A ($p<0.05$ and $p<0.001$, Student t test).

Serum IL-6 levels: In healthy controls (n=10) serum IL-6 levels were below 25pg/ml. Figure 1 shows serum IL-6 levels in healthy controls, Group B (inflammation), before and after treatment of infections, and Group C (LSIL), after treatment of infections.

Serum IL-6 levels (Mean±SEM) in both Groups B and C were higher than in healthy controls. In Group B mean serum IL-6 levels were 15.9±17.05pg/ml and 14.2±8.44pg/ml before and after treatment respectively ie within the normal range (Not significant vs Group A, and Before vs After treatment; Student t test).

After treatment of nonviral infections serum IL-6 levels were significantly high (86±122.4pg/ml, range 0-352 pg/ml) in women with persistent LSIL (Group C vs Group B, $p<0.05$;Group C vs healthy controls, $p<0.05$; Student t test). Eight cases of persistent LSIL had serum IL-6 below 10 pg/ml, whilst the remaining 5 had levels from 54-352pg/ml).

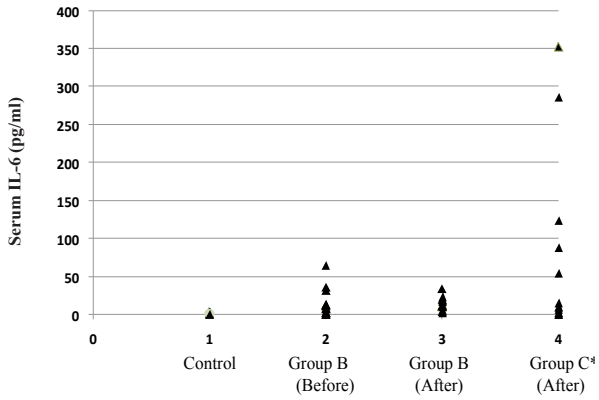

Figure 1. Serum IL-6 Level in Healthy Controls (n=10), Group B (N=14) Before & After Treatment, and in Group C (N=13) After Treatment of Infections
Student t test :* $p < 0.05$, Group C vs Group B and Controls

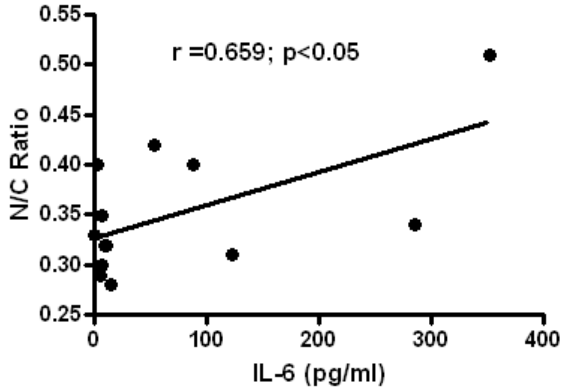

Figure 2. Correlation Between serum IL-6 Levels and N/C Ratio in Women with Pap Smears with Persistent LSIL

Correlation between serum IL-6 levels and N/ C ratio

Pearson's Coefficient of Correlation (r) between nuclear diameter and N/C ratio with serum IL-6 levels was calculated within each group. There was no correlation between IL-6 levels and nuclear diameters or N/C ratio in Group A and Group B (before or after treatment). There was a positive correlation between N/C ratio and IL-6 levels in Group C (LSIL) as seen in Figure 2 ($r = 0.659$; $p < 0.05$).

Discussion

In future immunization with HPV vaccine is likely to reduce the burden of cervical cancer (Basu, 2009), however until it becomes universal and long term efficacy is shown there have to be interim goals of cervical cancer prevention. Moreover the vaccine likely to prevent only about 70% of cervical cancers. Several thousands of women are already infected with various High Risk HPV subtypes and these are likely to progress to cervical cancer. Pap smear, conventional or Liquid Based Cytology, although rapidly being replaced by HR HPV DNA testing for screening, as technology advances, is still required when the test is positive (Bethesda System, 2001; Mali et al., 2004; Moscicki et al., 2004; Weinberg, 2007). When the report is LSIL, we know that often the lesion is reversible and that these women should undergo repeat smears and / or colposcopy. In India the estimated number of new cancers of cervix uter is 134,420 per year (Globocon, 2008), with an estimated >5,00,000 new cases of LSIL. This is a huge number to be followed up with a gynaecological check up in a country with limited resources. Moreover LSIL usually occurs in relatively younger age group hence conservative follow up takes precedence over surgical methods, which are not without recurrences. Since it is difficult to predict which women are more likely to develop cancer, in the present study an attempt is made to determine if circulating level of the pro-inflammatory cytokine IL-6 is high in women with Low-Grade Squamous Intraepithelial Lesions even after treatment of non-viral infections.

Whilst histopathological confirmation with a cervical biopsy is ideal, it is expensive and requires a gynaecologist and it cannot be repeated as it is traumatic. Hence it is important to determine a biomarker which can be assessed repeatedly without invasive/ surgical procedures. The local tissue (cervical biopsy) and vaginal fluid levels of IL-6 are reported to be high in cervical precancer and cancer.

Fluid from cervicovaginal washings can be collected only by special collection devices which are not universally available and the method for collection as well as assay has to be rigorously standardised.

IL-6 has a putative role in cancer and precancer (Tjiong et al., 1999; Salgado et al., 2002; Pardo-Govea et al., 2005; Heikkilä et al., 2008) hence we wanted to investigate the effect of treatment of lower genital infections, which are co-factors for carcinogenesis, on the serum IL-6 levels. We correlated the levels of serum IL-6 with the micrometric changes in the Papanicolaou smears repeated after treatment.

This study reveals that the serum IL-6 levels were markedly high in 5/13 cases in the LSIL group even after treatment of nonviral infections and serum IL-6 correlated positively with N/C ratio within the LSIL group. The mean levels of IL-6 were significantly high in the LSIL group C as compared to healthy controls. There is marked individual variation in IL-6 levels in LSIL. Indeed 8 cases had serum IL-6 level below 10 pg / ml. This may be dependent on genetic predisposition due to IL-6 polymorphism which may determine whether a case progresses to higher grades of neoplasia (Gangawar et al, 2009). Bustamam et al (2008) have used IL-6 as a systemic indicator in an experimental model in the rats and there is a need to investigate this further in large scale human studies. Whether higher levels indeed indicate a higher probability of progression can only be determined by long term follow up.

Limitations of the study: There was no histological confirmation of LSIL cases because in our setting biopsy is not carried out unless repeat Pap smear or colposcopy indicates HSIL or ASCUS-H. However the cytological criteria are well defined and described earlier. The LSIL group was included for serum IL-6 evaluation only after persistence of cytological features of LSIL in repeat Pap smears after treatment of infections.

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