A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions


Aims: To evaluate possible deregulation of the cytokine network by estimating the expression of peripheral cytokines in different stages of cervical disease and in relation to the presence or absence of high risk human papillomavirus (HR-HPV).

Methods: Twenty one HR-HPV positive women with high grade cervical intraepithelial neoplasia (CIN II–III) and 12 patients with invasive cervical carcinoma formed the study groups. Two control groups consisted of 10 HR-HPV positive and 11 HR-HPV negative women without CIN. Differences in leucocyte subgroups were evaluated by a differential leucocyte count. Plasma concentrations of tumour necrosis factor \( \alpha \) (TNF\( \alpha \)), TNF\( \alpha \) receptors TNFRI and TNFRII, interferon \( \gamma \) (IFN\( \gamma \)), interleukin 2 (IL-2), IL-12, IL-4, and IL-10 were determined by enzyme linked immunosorbent assays.

Results: Leucocyte counts in patients with CIN III and carcinoma were significantly higher than in controls. Plasma IFN\( \gamma \) concentrations were significantly lower in patients with CIN III and carcinoma than in women with CIN II or controls. Plasma concentrations of IL-12, IL-2, IL-4, and TNF\( \alpha \) did not differ significantly between groups, but significantly lower plasma concentrations of TNFRII were found in CIN III and carcinoma compared with CIN II. IL-10 was detected with increased frequency in the plasma of patients with CIN III and carcinoma.

Conclusions: These results indicate that a shift to a Th2-type cytokine pattern during the carcinogenesis of cervical cancer occurs in women with CIN III lesions.
controls. Cervical cytology, histology, and HPV sampling were obtained. Exclusion criteria for all participants were: postmenopausal state, pregnancy at time of sampling, chronic diseases (diabetes, allergy, or autoimmune disease), signs of acute infection at the time of sampling, and an immunocompromised state. Two women with allergy, one woman with an autoimmune disease, and one woman with an acute infection at the time of sampling were excluded. With the exception of oral contraceptives, patients did not use medication on a regular base. Pain medication (non-steroidal anti-inflammatory drugs) was omitted for at least two weeks before sampling.

Our study protocol was approved by the hospital and university ethics committee and all women voluntarily gave signed informed consent.

### Table 1: HPV typing in patients with cervical dysplasia (CIN II, CIN III, and carcinoma) and in HR-HPV positive controls

<table>
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### Figure 1: Differential white blood cell counts in patients with high risk human papillomavirus infection and cervical dysplasia and in high risk HPV negative and high risk HPV positive controls. Data are shown as mean ± SEM. *p < 0.05 when compared with CIN II. **p < 0.01 when compared with CIN II. ***p < 0.01 when compared with CIN III. CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

### Figure 2: Plasma concentrations of IFN-γ in high risk HPV positive patients with cervical dysplasia and in high risk HPV negative and high risk HPV positive controls. Median values are indicated by a horizontal line. CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; IFN-γ, interferon γ.

### HPV sampling and determination

Cervical scrapes for HPV detection and typing were taken by a cervical biosampler (Accellon Combi; Medscand Medical, Malmo, Sweden). HPV testing was performed with the consensus GP5+/GP6+ polymerase chain reaction (PCR) enzyme immunoassay for 37 (sub)types, as described previously. This test is clinically validated. We used β globin PCR to identify sampling errors and to monitor for PCR inhibitors. In addition, reverse line blot analysis was performed on PCR enzyme immunoassay positive cases to identify individual HPV types.

### Blood sampling and processing

Peripheral venous blood samples were collected between 8.00 and 12.00 a.m. in sterile endotoxin free vacutainers (Becton Dickinson, Meylan, France) with ethylenediaminetetraacetic acid (EDTA) as anticoagulant and immediately centrifuged at 1500 g for 10 minutes at 4°C. Plasma samples were stored at −80°C until assayed.

A differential leucocyte count was performed in all blood samples with a Sysmex XE-2100.

### Cytokine determinations

All plasma samples were analysed by commercially available enzyme linked immunoassays (Biosource Europe, Nivelle, Belgium) for the cytokines TNF-α, soluble TNF-α receptors (sTNFRI and sTNFRII), IFN-γ, IL-2, IL-4, IL-10, and IL-12. The detecting antibody in the immunoassay for IL-12 recognised the bioactive heterodimeric (p40+p35) cytokine, in addition to the p40 subunit homodimer. According to the manufacturer, the minimal detectable concentrations (MDCs) and intra-assay and interassay coefficients of variation (CVs) were as follows: TNF-α: MDC, 3 pg/ml; CVs, < 6% and < 10%; sTNFRI: MDC, 50 pg/ml; CVs, < 7% and < 10%; sTNFRII: MDC, 100 pg/ml; CVs, < 7% and < 10%; IFN-γ: MDC, 2 pg/ml; CVs, < 5% and < 10%; IL-2: MDC, 7 pg/ml; CVs, < 6% and < 10%; IL-4: MDC, 2 pg/ml; CVs, < 5% and < 10%; IL-10: MDC, 1 pg/ml; CVs, < 5% and < 10%; IL-12 + p40: MDC, 1.5 pg/ml; CVs, < 10% and < 10%.
sTNFRI and sTNFRII in high risk HPV positive patients with cervical squamous cell carcinomas, two adenocarcinomas. The control groups consisted of 11 women without cervical dysplasia without HR-HPV and 10 women without cervical dysplasia but with a positive HR-HPV test. The mean (SD) age was 32.4 (6.2) years for the HR-HPV positive controls, 27.9 (7.4) years for the HR-HPV negative controls, 32.3 (5.5) years for patients with CIN II, 32.4 (6.2) years for the HR-HPV negative controls, 33.4 (5.7) years for the HR-HPV positive controls, but no other significant changes in IL-2 values. IL-12 showed no significant differences between patient groups.

To calculate cytokine ratios, values below the detection limit were assigned concentrations equal to one half of the lower detection limit of each assay.

Statistical analysis

Cytokine data are presented as medians with ranges unless stated otherwise. The non-parametric Kruskal-Wallis test and Mann-Whitney U test were used as appropriate to assess differences in cytokine values between groups, and Spearman’s rank test was used to investigate correlations between cytokine variables. A two tailed p value of 0.05 was chosen to represent significance. Differences in leucocytes and leucocyte subpopulations were evaluated by unpaired t tests.

RESULTS

The study groups

Our study group comprised 33 women: 10 women with moderate dysplasia (CIN II), 11 women with severe dysplasia (CIN III), and 12 women with cervical carcinoma (10 squamous cell carcinomas, two adenocarcinomas). The control groups consisted of 11 women without cervical dysplasia without HR-HPV and 10 women without cervical dysplasia but with a positive HR-HPV test. The mean (SD) age was 32.4 (6.2) years for the HR-HPV negative controls, 27.9 (7.4) years for the HR-HPV positive controls, 33.4 (5.7) years for patients with CIN II, 32.3 (5.5) years for those with CIN III, and 35.6 (7.2) years for women with invasive carcinoma.

Results of HPV determinations

HPV types 16, 18, and 31 were the most frequently detected HR-HPV types (n = 18, n = 7, and n = 8, respectively). Other HR types were less common: HPV-33, n = 2; HPV-35, n = 1; HPV-39, n = 2; HPV-43, n = 1; HPV-51, n = 1; HPV-52, n = 2; HPV-56, n = 6; HPV-58, n = 1; HPV-59, n = 2; and HPV-82, n = 1. HPV-70 (low risk type) was detected together with HPV-16 in one woman with CIN III and as the single infecting agent in one of 12 patients with carcinoma. There were 34 single and eight multiple HPV infections. Table 1 summarises the HPV types within the study groups.

The differential white blood cell count

The total leucocyte count was higher in patients with CIN III and carcinoma than in CIN II and the control groups; the difference between CIN II and CIN III was significant. The neutrophil count showed a similar course, with a significant increase from CIN II to carcinoma. Absolute monocyte and lymphocyte counts varied significantly between groups, being highest in women with CIN III. Figure 1 shows the results of the differential white blood cell counts.

The cytokine profile

The results of cytokine assays were calculated for each 10^6 leucocytes, to stratify for possible different numbers of cytokine producing leucocytes between study subjects.

Analysis of the peripheral cytokine profile in our study groups revealed different courses for each of the eight investigated cytokines. Differences between groups were seen for IFNγ concentrations, with a significant decrease from CIN II to CIN III and carcinoma (p ≤ 0.01), as summarised in fig 2.

There were no significant differences in plasma concentrations of TNFα between the control and study groups. Both TNF receptors were significantly correlated in all groups (r = 0.973, p = 0.000 for HR-HPV negative controls; r = 0.855, p = 0.002 for HR-HPV positive controls; r = 0.979, p = 0.001 for patients with CIN II; r = 0.791, p = 0.004 for patients with CIN III; and r = 0.748, p = 0.005 for patients with invasive carcinoma). TNFRII values were highest in HR-HPV positive controls. A significant decrease was seen in patients with cervical carcinoma compared with patients with CIN II. A similar pattern was seen for TNFRα but the differences did not reach significance. Figure 3 shows the median plasma concentrations of TNFα and its two soluble receptors sTNFRI and sTNFRII in all study groups.

Table 2: Plasma concentrations of IL-4 and IL-10 in HR-HPV positive patients with cervical dysplasia and in HR-HPV negative and positive controls

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>IL-4 &gt; detection limit* in x/N plasmas</th>
<th>IL-10 &gt; detection limit* in x/N plasmas</th>
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<tr>
<td>HR-HPV– controls</td>
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<td>2/11</td>
<td>2/11</td>
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<tr>
<td>HR-HPV+ controls</td>
<td>10</td>
<td>1/10</td>
<td>0/10</td>
</tr>
<tr>
<td>HR-HPV+ CIN II</td>
<td>10</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td>HR-HPV+ CIN III</td>
<td>11</td>
<td>1/11</td>
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</tr>
<tr>
<td>HR-HPV+ carcinoma</td>
<td>12</td>
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<td>7/12</td>
</tr>
</tbody>
</table>

*Detection limit for IL-4, 2 pg/ml; detection limit for IL-10, 1 pg/ml.

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HR, high risk; IL, interleukin.
groups. IL-4 concentrations were below the detection limit in most of the investigated plasma samples. IL-10 values were below the detection limit in most control women and women with moderate dysplasia, but were detectable in four of 11 patients with CIN III and seven of 12 invasive carcinomas. Table 2 shows the data for IL-4 and IL-10.

A possible shift to increased expression of Th2-type cytokines in patients with carcinoma was evaluated by calculating the ratio of Th1-type cytokines IL-12, IFN\(\gamma\), TNF\(\alpha\), and IL-2 to the Th2-type cytokine IL-10 for all study groups. These ratios decreased significantly \((p < 0.01)\) for IFN\(\gamma\) : IL-10, \(p = 0.02\) and \(p < 0.01\) for TNF\(\alpha\) : IL-10, \(p = 0.03\) and \(p = 0.06\) for IL-2 : IL-10) when patients with carcinoma were compared with HR-HPV positive controls or patients with CIN II. Figure 4 shows as an example the ratio of IL-12 : IL-10.

**DISCUSSION**

Our data for the differential white blood cell counts in our patients are in agreement with the results of Onsrud et al, who reported increased leucocyte and neutrophil counts in cervical carcinoma, probably induced by inflammation and necrosis, and a decrease in absolute lymphocyte numbers in comparison with healthy controls. Studies by Gemignani et al and Balaram et al confirm these results. Stratification of cytokine data for each 10\(^{4}\) leucocytes seems justified.

Our study showed a significant decrease in circulating IFN\(\gamma\) concentrations in women with severe dysplasia and invasive carcinoma. IFN\(\gamma\), secreted by Th1 cells, cytotoxic T cells, and stimulated natural killer cells, is a major contributor to an effective Th1-type cellular immune response against HPV infections. Defective IFN\(\gamma\) production may be associated with persistent HPV infection and the development of HPV related neoplasia. Our data for circulating IFN\(\gamma\) agree with these reports.

TNF\(\alpha\) has been shown to act synergistically with IFN\(\gamma\) on cervical cancer cells by inducing apoptosis or necrosis. The biological effects of TNF\(\alpha\) are mediated by its two receptors TNFRI and TNFRII. Both receptors are present on many cell types and tissues; TNFRI is strongly expressed on cells that are sensitive to the cytotoxic actions of TNF\(\alpha\), whereas TNFRII is strongly expressed on stimulated T and B cells. Several agents including TNF\(\alpha\) itself downregulate surface expression of both receptors by proteolytic cleavage of the extracellular domains that are shed as soluble proteins—sTNFRII and sTNFRI—into the circulation. Measurement of both soluble receptors, especially of sTNFRII, is often used to assess Th1-type activity in cell mediated immune responses. Our study showed no significant differences in TNF\(\alpha\) values between study groups. However, there was a decrease in both soluble receptor concentrations in the plasma of patients with carcinoma, which reached significance for sTNFRII. This result reflects a decrease in circulating TNF\(\alpha\) and is in accordance with a disturbed Th1-type immune response after progression to CIN III and invasive carcinoma.

There were no significant differences in peripheral concentrations of the Th1-type cytokine IL-2 between our study groups, except a slight increase \((p = 0.037)\) between HR-HPV negative and HR-HPV positive controls, which could indicate an inflammatory response to the viral infection.

A possible shift to Th2-type cytokines in the course of development of cervical neoplasia may be reflected by increased plasma concentrations of Th2-type cytokines IL-4 and IL-10. However, we found no significant differences in IL-4 concentrations between our study groups. IL-10 concentrations increased significantly with the severity of dysplasia. IL-10 is a potent immunosuppressive cytokine, which inhibits T cell activation and Th1 cell differentiation. This shift to Th2-type activity was characterised by Jacobs et al as a significant decrease in the IL-12 : IL-10 ratio in patients with high grade squamous intraepithelial lesions. IL-12, a key cytokine in the induction of cell mediated immunity, promotes T cell differentiation into IFN\(\gamma\) producing Th1 cells and induces IFN\(\gamma\) production in NK cells. The significant decrease in IL-12, IFN\(\gamma\), TNF\(\alpha\), and IL-2 relative to IL-10 in patients with CIN III and carcinoma, as seen in our study, supports and extends the finding of Jacobs et al of a disturbed cellular immune response in the development of severe cervical dysplasia and cervical carcinoma. The presence of diseases that could influence circulating cytokine concentrations was excluded in all participants, so that presumably these results are the consequence of HPV infection.

The changes that we found within the peripheral cytokine network in cervical dysplasia and invasive cancer were not

**Take home messages**

- We found significant decreases in circulating interferon \(\gamma\) and tumour necrosis factor \(\alpha\) receptor II and an increase in interleukin 10 concentrations through the different stages of dysplasia to invasive carcinoma
- These results indicate a shift towards a Th2 helper 2-type (Th2) cytokine pattern during the carcinogenesis of cervical cancer in women with cervical intraepithelial neoplasia (CIN) III lesions
- Vaccinations aimed at converting the Th2 response into a Th1 response might be useful in the treatment of patients with CIN II and III
always in agreement with published data for individual cytokines. In a study by Niwa et al., IFNγ concentrations in plasma from cervical cancer and controls were similar, whereas in a study by Lebrecht et al., IFNγ concentrations were below the detection limit in all groups. Our results for plasma concentrations of TNFRI were in accordance with the results of Sheu et al., who found lower serum concentrations of sTNFRI in patients with cervical cancer when compared with women with a benign disease, uterine myoma. However, they differ from the results of Malejczyk et al., in whose study patients with HPV-16 associated CIN I/II and CIN III lesions and with HPV-16/18 associated squamous cervical cancer were compared with healthy controls. There were no significant differences in receptor values between women with CIN lesions and controls, but both receptor concentrations increased significantly in patients with cervical cancer. Important factors that contribute to differences in results compared with earlier studies include selection criteria for controls and patients. In our study, we selected patients and controls according to their state of infection with HR-HPV. The single presence of low risk HPV type 70 in one woman with cervical carcinoma could be explained by HR-HPV DNA integrated via the L1 region in the cellular genome, which can be missed by the GP5+/6+ PCR. With the exception of the studies of Jacobs et al. and Malejczyk et al, none of the above mentioned studies investigated the presence of HPV. Moreover, stratification for leucocyte numbers might also explain these discrepancies. Immunocompetent leucocytes are the main producers of circulating cytokines. In contrast to all other studies, we calculated our results according to the leucocyte count and not for each milliliter of plasma. Finally, analytical methods for the measurement of cytokines may differ. Some studies used plasma, others serum, a choice that influences the quantitative outcome (unpublished results, 2005). All studies used enzyme linked immunoassays, which were produced by different manufacturers. This implies the use of different antibody systems for cytokine binding and quantification with different sensitivities.

Our results indicate a change to a Th2 cytokine pattern already manifest in women with CIN III lesions. This provides the theoretical basis of the clinical policy to treat all women with CIN III lesions. Furthermore, it might indicate that, in patients with cervical cancer, vaccinations should be directed towards conversion of the Th2 response into a Th1 response, and that they may also be useful in women with CIN II lesions.

In conclusion, our study showed characteristic changes within the peripheral cytokine network during the course of HR-HPV infection through different stages of dysplasia to invasive carcinoma: a significant decrease in circulating IFNγ and sTNFRI and an increase in IL-10 concentrations. This indicates a shift towards a Th2-type immune response in the development of cervical cancer. The changes in cell mediated immunity become manifest in patients with CIN III, which is in line with the clinical observation that most CIN III lesions do not regress spontaneously, and may ultimately progress into invasive carcinoma if not properly treated.

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J Clin Pathol 2005 58: 1096-1100
doi: 10.1136/jcp.2004.025072