Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression

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SUMMARY

We have addressed the notion that the progression of cancer of the uterine cervix is associated with a preferential constraint on the development of a type 1 cellular mediated response, which is necessary to efficiently eliminate (pre)neoplastic cells. Based on the importance of cytokines in the regulation of an appropriate immune response, we have evaluated the expression of IL-12p40, IL-10 and transforming growth factor-beta 1 (TGF-β1). Using reverse transcriptase-polymerase chain reaction (RT-PCR), the expression of these three cytokines was evaluated in both low-grade (LG) and high-grade (HG) cervical squamous intraepithelial lesions (SIL) and in normal exocervix and transformation zone biopsies. Our results show that the average level of IL-12 increases within both the LG and HG SIL, compared with both control groups. Interestingly, the percentage of HG SIL expressing IL-12p40 was lower compared with LG SIL. In contrast, the expression of IL-10 increased in parallel with the severity of the lesion to a maximal level in HG SIL. Using immunohistochemistry, we ascertained the presence of IL-12 protein in SIL and IL-10 protein in the transformation zone and SIL biopsies. Both IL-12- and IL-10-producing cells were localized in the stroma, not within the SIL. Furthermore, in this study we also observed that the region of the cervix the most sensitive to lesion development, the transformation zone, was associated with higher average levels of the immunosuppressive cytokines IL-10 and TGF-β1.

Keywords   IL-12   IL-10   transforming growth factor-beta 1   squamous intraepithelial lesions   local immunity

INTRODUCTION

The chronic infection of keratinocytes of the uterine cervix by the human papilloma virus (HPV) is associated with the development of cervical cancer [1]. Despite the evidence that HPV is strongly implicated as the causative agent in the aetiology of cervical cancer and its precursors (squamous intraepithelial lesion (SIL)), HPV infection alone is not sufficient for cancer development [2]. Following the onset of an initial HPV infection, the progression to SIL and to cancer can be divided into several distinct stages. Accordingly, cervical cancer represents an excellent model system for studying the interactions between cells transformed by an oncogenic agent and the immune system during the progression of the SIL.

The fact that only a small proportion of HPV-infected individuals will eventually develop cancer of the cervix and the long latency period between primary infections and cancer emergence suggest that additional factors are involved in the progression of the SIL. A substantial majority of SIL and cancers develop within a specific region of the cervix, the transformation zone, implying that other exogenous or endogenous factors specific to the anatomical milieu may be conducive to SIL and cancer development. Likewise, the augmented frequency of SIL and cervical cancers in AIDS patients suggests the importance of the immune response, and more specifically T lymphocytes, in the prevention or limitation of HPV-associated lesions [3]. Moreover, several studies have shown that antibody-mediated immune responses to HPV reflect more the tumour progression than a protective host mechanism against development of disease [4,5]. In contrast to a cell-mediated immune response, this inefficient antibody-mediated response may be permissive to tumour progression [6].

It is well established that immune responses can be principally divided into a type I cellular-mediated response or a type II antibody-mediated response. The initiation and maintenance of a type I or type II response is dependent on many factors, including the type of antigen-presenting cell (APC), costimulatory molecules, the type and concentration of antigen and the cytokine milieu [7]. These two responses are associated with either CD4 T helper cells that produce IL-2, interferon-gamma (IFN-γ) and

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tumour necrosis factor-beta (TNF-β) (type I), or IL-4, IL-5, IL-6 and IL-13 (type II) [8]. In addition, several cytokines have been shown to contribute to the initiation or suppression of these immune responses, such as IL-4, IL-12, IL-10 and/or transforming growth factor-beta 1 (TGF-β1) [9–12]. These cytokines have been shown to be produced by various cell types, including macrophages, dendritic cells and keratinocytes [13–15].

It has been shown that peripheral blood mononuclear cells (PBMC) from patients with both SIL and cancer produce decreased amounts of IL-2 and IFN-γ and higher levels of IL-4 and IL-10 following mitogenic stimulation, compared with the control group [16]. Recently, our group has demonstrated that basal levels of IL-10 are augmented in the PBMC of patients with SIL [17]. Studies by our group and others, focused on the status of the localized immune response, have shown that SIL and/or cancer are associated with elevated levels of IL-4 and IL-6 [18,19]. Moreover, we have also shown that high-grade (HG) SIL are associated with lower densities of IL-2-producing cells compared with normal biopsies [19]. Taken together, these results argue that the development of SIL and/or cervical cancer is preferentially associated with type II (IL-4/IL-6) or immunosuppressive (IL-10) cytokines, as has been demonstrated in other types of cancers [20–23].

As an approach to understanding the factors involved in the generation and maintenance of an inefficient anti-tumour response, we have evaluated the expression of three cytokines, IL-12, IL-10 and TGF-β1, known to play an important immunomodulatory role. In this study, we used the techniques of reverse transcriptase-polymerase chain reaction (RT-PCR) and/or immunohistochemistry to analyse the expression of these cytokines in normal exocervix, transformation zone and in SIL biopsies.

MATERIALS AND METHODS

Biopsies
Forty-three cervical biopsies (2–3 mm) were analysed in this study. Normal biopsy material was obtained from women undergoing routine examinations or hysterectomies. Biopsies from women with SIL were obtained before surgical procedures. Biopsies were immediately frozen in Tissue-Tek (Miles, Elkhart, IN) and stored at −80°C. For each biopsy the histology was assigned to one of four categories based on histologic findings after haematoxylin–eosin staining: (i) normal exocervix from healthy women; (ii) transformation zone from healthy women; (iii) low-grade (LG) SIL including condyloma and cervical intraepithelial neoplasia (CIN) I lesions; (iv) HG SIL, including CIN II and III.

Preparation of nucleic acids
DNA and total RNA were prepared from all biopsies. To detect the presence of HPV, crude DNA was prepared from frozen biopsy sections (10 μm) by digesting the tissue sections with proteinase K (1 mg/ml) (Boehringer, Mannheim, Germany). RNA was extracted from 40 sections (10 μm) of frozen biopsies using the guanidinium thiocyanate method (RNAzol B; Bioprobe, Montreuil, France).

PCR and RT-PCR
PCR of DNA was carried out using PCR Master (Boehringer) with a standard aliquot of the DNA preparations. PCR of β-actin and L_1 HPV genes was amplified for each sample using published oligonucleotide sequences [24]. The cDNA for the RT-PCR was prepared using M-MULV reverse transcriptase and 250–500 ng of total RNA from each biopsy, using standard conditions

recommended by the vendor (GIBCO/BRL, Merelbeke, Belgium). The PCR was carried out using standard aliquots of cDNA with: 1·25 U of Taq 2000 (Stratagene, La Jolla, CA), 0·2 mM dNTPs and 20 pg of each primer pair (HPRT sense GTTGGTATAAGCCAGA CTTTGTTG, antisense CAGATTTTCCAAACTCAACTTGAA, TGF-β1 sense TGAGGCCGACTACTACGCCAA, antisense GA GCCCTGGACACCAACTATT, IL-10 sense ATGCTTCGAGAT CTCCGAGA, antisense AAATCATACACGCCGTA, IL-12p40 sense ATTGAGGTCATGGTGGATGC, antisense AATGCTGG CATTTTTGCGGC and IFN-γ sense GCAGAGCCAAATTGTC TCCT, antisense ATGCTCTTCGACTCGAAAC). All primer pairs spanned at least one intron. Cycling conditions were: 95°C 5 min/55°C or 60°C 1 min/72°C 1 min (35–40 cycles). A negative control without cDNA was included for each PCR reaction. The results are expressed as semiquantitative values based on the ratio of the intensity of the cytokine and HPRT PCR products analysed on ethidium bromide- or SYBR Green (Molecular Probes, Eugene, OR)-stained agarose gels (1·8%) using the BIO-PROFIL gel analyser system (Vilber Lourmat, Marne La Vallée, France). The specificity of all PCR products was verified using the Southern blot technique with ^{32}P-labelled internal oligonucleotides. Statistical evaluation of the PCR results was done using the Mann–Whitney test (InStat; Graph Pad Software, San Diego, CA).

Immunohistochemistry for IL-10 and IL-12
Frozen sections (8 μm) were fixed in 2% paraformaldehyde and permeabilized using 0·1% saponin (UCB, Louvain, Belgium). Endogenous peroxidase was blocked with 0·2% H_2O_2 in PBS/ 0·1% saponin for 30 min. The slides were then incubated overnight at 4°C with the cytokine-specific MoAbs (IL-12, Medgenix, Fleurus, Belgium; IL-10, Medgenix or Pharmingen, San Diego, CA) or isotype controls in PBS/0·1% saponin/2% bovine serum albumin (BSA)/0·1% NaN$_3$. The anti-IL-12 antibody recognizes both IL-12p40 and IL-12p70. Biotin-labelled secondary antibodies (1:100 biotin goat anti-mouse IgG, Vector Labs, Burlingame, CA; 1:100 biotin-goat anti-rat, Pharmingen) were applied for 30 min at room temperature. The slides were then incubated with avidin–biotin–horseradish peroxidase (HRP) complex (Vectastain, ABC-HRP kit; Vector) for 30 min. Diaminobenzidine (0·5 mg/ml; Sigma, Bornem, Belgium) was used as chromogen. Slides were counterstained with haematoxylin and mounted for light microscopy.

RESULTS

Expression of IL-12, IL-10 and TGF-β1 in SIL
We have used the technique of RT-PCR to evaluate the expression of cytokines that could play a positive (IL-12) or negative (IL-10/ TGF-β1) role in the generation or maintenance of a cellular immune response. Included in our series of biopsies were: normal exocervix, the transformation zone, LG SIL and HG SIL. In Fig. 1 the data from the RT-PCR experiments are expressed as the relative level of cytokine expression (left ordinate) and as the percentage of biopsies expressing each cytokine (right ordinate). The relative expression level of IL-12p40 was higher in both LG and HG SIL compared with both normal exocervix ($P = 0·01/0·08$, respectively) and transformation zone biopsies ($P = 0·08/0·39$, respectively). The percentage of biopsies expressing IL-12p40 peaked in the LG SIL at 83% and diminished to 63% in HG SIL. In contrast, the expression of IL-10 increased continuously from a relatively low level in the normal exocervix to the highest average level and percentage in HG SIL ($P = 0·0004$). Interestingly, the
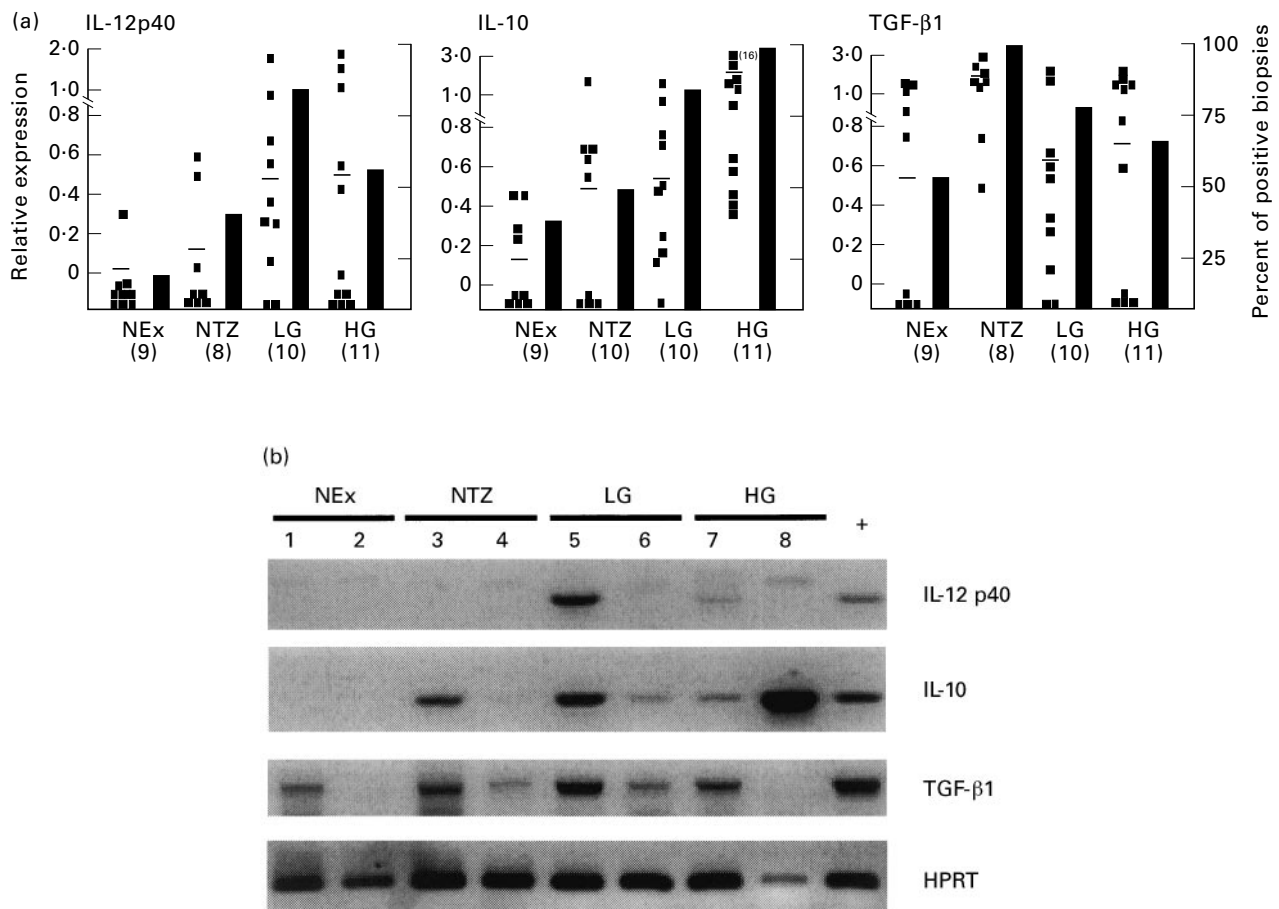
Fig. 1. Increased expression of IL-12p40 and IL-10 in squamous intraepithelial lesions (SIL). A group of 43 biopsies was evaluated for the expression of several cytokines using reverse transcriptase-polymerase chain reaction (RT-PCR). (a) The relative values of cytokine expression for IL-12, IL-10, transforming growth factor-beta 1(TGF-β1) *versus* HPRT. The left ordinate represents the average relative level of expression and the right ordinate represents the percentage of biopsies expressing the cytokine. Each point represents one patient and the average expression level is indicated by a bar. (b) Example of PCR products visualized on ethidium bromide (TGF-β1 and HPRT)- and SYBR Green (IL-10 and IL-12)-stained gels. NEx, Normal exocervix; NTZ, normal transformation zone; LG, low-grade SIL; HG, high-grade SIL; +, tonsil positive control. All normal biopsies were human papillomavirus (HPV)-negative and all SIL were HPV-positive.

percentage of positive biopsies and the average expression level of IL-10 was higher in the transformation zone compared with the exocervix. Figure 1b is a representative illustration of a gel electrophoresis of RT-PCR products derived from eight of the biopsies analysed. Contrary to the differential expression of IL-10 and IL-12p40, IFN-γ was expressed by a vast majority of the biopsies and the average expression levels were similar for all groups (data not shown).

To complement this work we analysed several biopsies using immunohistochemistry to demonstrate the presence and the localization of the cytokine proteins. We found that the density of IL-12-producing cells was higher in SIL biopsies compared with normal exocervix biopsies (Fig. 2). Compared with HG SIL, a higher percentage of LG SIL were positive for IL-12-producing cells (data not shown). IL-10-producing cells were rare (Fig. 3), but they were more frequently found in the transformation zone and SIL biopsies compared with the normal exocervix. Both IL-10- and IL-12-positive cells were found within the stroma, not within the SIL.

TGF-β1 (Fig. 1) was expressed at similar levels in the exocervix biopsies and SIL. In contrast, the expression of TGF-β1 was

highest in the transformation zone, where 100% of the biopsies expressed the cytokine. Compared with the biopsies from the transformation zone, the average level of TGF-β1 expression diminished in SIL (LG $P = 0.05$, HG $P = 0.03$).

Differential expression of IL-10 in the transformation zone
The region most sensitive to SIL and cancer development is the transformation zone. Since we found that the transformation zone biopsies expressed higher average levels of IL-10 than exocervix biopsies, we wanted to evaluate the expression of this cytokine using both biopsies derived from individual patients. Figure 4 shows the results of a gel electrophoresis analysis of IL-10 expression in three individuals. We observed that in two out of the three patients (66%) analysed the transformation zone was associated with higher expression levels of IL-10.

DISCUSSION

In this study we have demonstrated that the progression of SIL is associated with a locally augmented expression of IL-10, an immunosuppressive cytokine. Coincident with the expression of
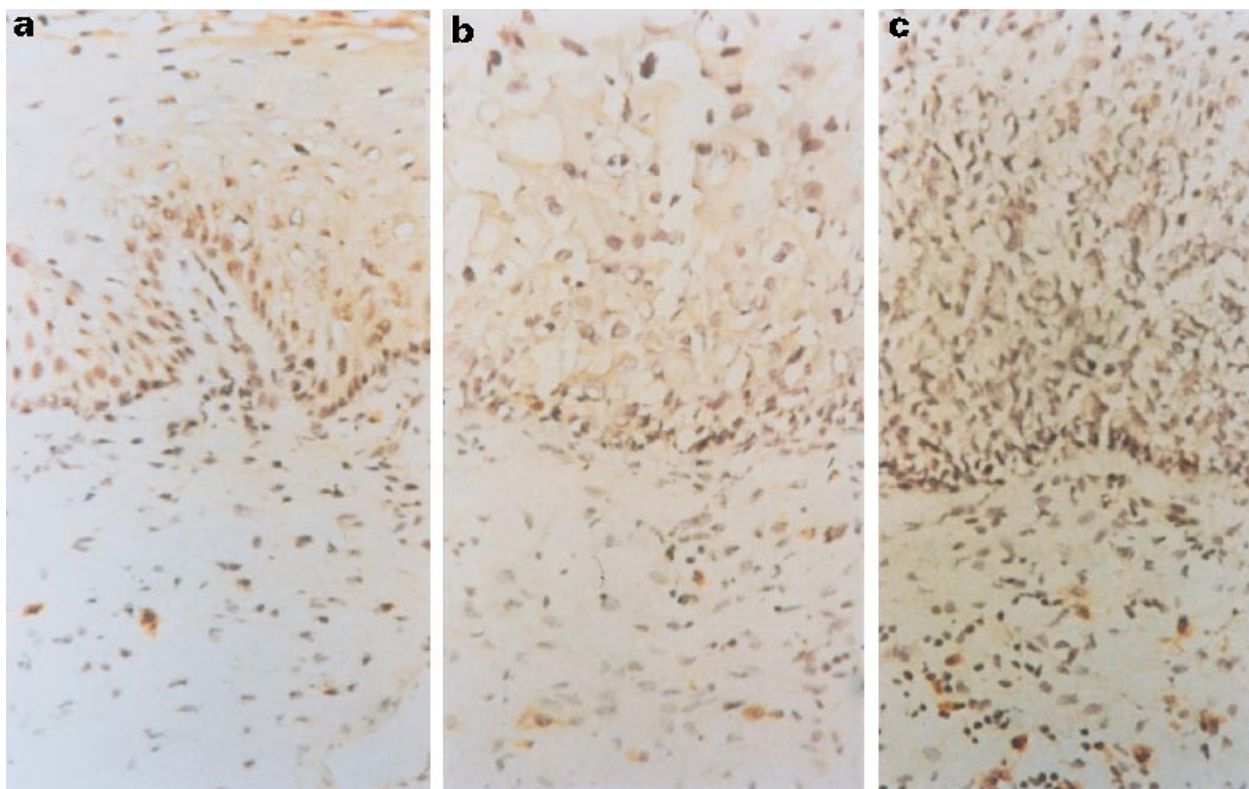
Fig. 2. Detection of IL-12 protein in squamous intraepithelial lesions (SIL). Immunohistochemical staining for IL-12: (a) normal exocervix, (b) low-grade SIL, and (c) high-grade SIL. Objective: ×40.
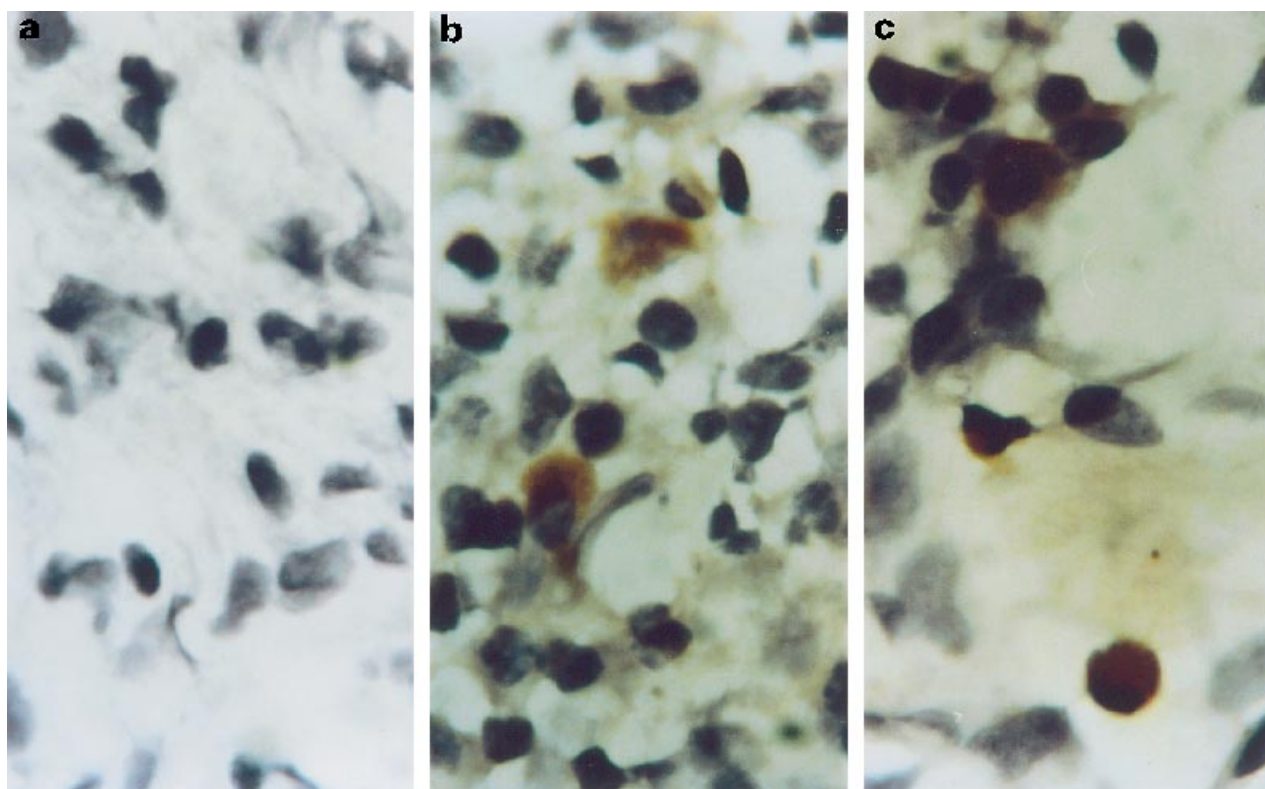
Fig. 3. Detection of IL-10 protein in squamous intraepithelial lesions (SIL). Immunohistochemical staining for IL-10: (a) normal exocervix, (b) transformation zone, and (c) SIL. Objective: ×100 oil immersion.
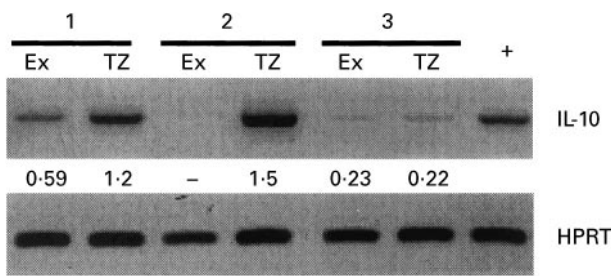
Fig. 4. Differential expression of IL-10 in the transformation zone (TZ) and exocervix (Ex). Example of polymerase chain reaction (PCR) products visualized on ethidium (HPRT)- and SYBR Green (IL-10)-stained gels. The relative expression values are indicated below each cytokine band.

IL-10, the average levels of IL-12p40 also increased in SIL. Interestingly, we observed that the percentage of HG SIL expressing IL-12 declined compared with LG SIL, using both the technique of RT-PCR and immunohistochemistry. Thus the possibility exists that the loss of IL-12p40 expression in some HG SIL and the maintenance of IL-10 expression contributes to an efficient tumour escape mechanism. Moreover, we have observed that the transformation zone of the cervix, the region most sensitive to SIL and cancer development, is associated with higher average levels of the immunosuppressive cytokines IL-10 and TGF-β1. Since both cytokines have the ability to interfere with the efficient induction of a type I response by APC, these cytokines may contribute to the predisposition of this region to cervical carcinogenesis.

Fundamental to the efficacy of IL-12 as a potent cytokine is its ability to induce IFN-γ production in T and natural killer (NK) cells and the differentiation and expansion of naive T cells into Th1 cells [7,25,26]. Apart from the importance of IL-12 in the defence against intracellular pathogens such as viruses, several studies have shown that IL-12 exhibits anti-tumour activity in a variety of murine tumour models, including melanoma, bladder, colon and renal carcinoma, as well as in HPV-associated transplanted tumours ([27–29], Hallez, personal communication). IL-12 has also been shown to play a role in the inhibition of angiogenesis, a process important for tumour survival and metastasis [30]. Importantly, we observed the expression of IL-12p40 within the SIL, albeit 37% of HG SIL did not express detectable levels of the IL-12p40. The lack of potential IL-12 in some HG SIL may predispose the progression of HG SIL to cancer. The relevance of the increased expression of IL-12p40 in SIL needs to be interpreted in the context of the complex regulation of bioactive IL-12, which is formed by the association of IL-12p35 and IL-12p40 subunits. Based on the reported constitutive expression of IL-12p35 [31], the potential to form bioactive IL-12p70 should exist in SIL that produce IL-12p40 protein. However, since very sensitive techniques such as bioassays and ELISAs are recommended to detect the limited levels of IL-12p70, we were unable to assay for IL-12p70 in our biopsy specimens. Moreover, it is also known that the formation of IL-12p40 homodimers, which bind to the IL-12 receptor, can interfere with IL-12 bioactivity [32]. Thus, although the potential to form bioactive IL-12p70 exists in SIL, the possible formation of IL-12p40 homodimers may interfere with the activity of IL-12p70 to influence the local immune response.

In contrast to IL-12, IL-10 is known to have immunosuppressive effects resulting from the down-regulation of CD80 and MHC II molecules, which are necessary for efficient antigen presentation

[13,33]. Moreover, IL-10 inhibits the production of IL-12 *in vitro* [34,35]. Another study has shown that IL-10 can also interfere with the cytotoxic T lymphocyte (CTL) lysis of tumours [36]. In contrast to what is observed in mice, IL-10 can not be strictly associated with a type II response and has been shown to be produced by both type 1 and type 2 human T cells [37,38]. IL-10 mRNA and/or protein have been found to be augmented in several human cancers, such as renal and ovarian cancer and squamous and basal cell carcinoma of the skin [22,23,39,40]. In some cases IL-10 has been shown to be produced by the tumour cells themselves [23,41]. In addition, *in vitro* experiments have established that tumours can induce the production of IL-10 by PBMC [20,42]. In SIL, we observed that IL-10 was produced by stromal cells and not by preneoplastic cells within the SIL, as we previously observed for IL-4-producing cells [19]. The observed preferential expression of IL-10 in the transformation zone in some patients may contribute to the initiation of SIL by allowing HPV to subvert innate immunological surveillance mechanisms. Subsequently, the persistence of IL-10 in SIL may tolerize the immune system and permit the lesion to progress to cancer.

The association of an augmented level of both IL-12 and IL-10 in some SIL may be related to the recent observation of *in vitro* experiments demonstrating that IL-12 induces the augmentation of IL-10 and IFN-γ in human T cells [43,44]. However, in our experimental group we did not observe any correlation between the relative expression levels of IL-10 and IL-12 in SIL. Despite the expression of IL-10 in SIL, the expression pattern of IFN-γ was similar in all experimental groups (data not shown). This may be relevant to the observation that IL-10 has been shown to suppress the production of IFN-γ by type 1 CD4 cells and not by NK cells [45,46]. The significance of the concomitant expression of both IL-12 and IL-10 *in vivo* within the microenviroment of a preneoplastic lesion or tumour is unknown.

In cervical carcinogenesis TGF-β1 can play two contrasting roles. It may contribute to immunosuppression by down-regulating IL-2 receptor signalling in T cells and IL-12 expression by APC and by inducing the expression of IL-10 by macrophages [47–49]. In contrast, TGF-β1 can also be beneficial, in that it has been shown *in vitro* to inhibit the proliferation of keratinocytes and the expression of the E6 and E7 genes of HPV essential for cervical carcinogenesis [50,51]. Our results show that the overall expression of TGF-β1 is similar in the normal exocervix and SIL. In fact, a recent study has demonstrated that TGF-β1 is expressed in both normal exocervix and SIL, but that in the normal exocervix the expression is predominately in the epithelium and in SIL biopsies predominately in the stroma [52]. Surprisingly, we have observed that the transformation zone expressed the highest average levels of TGF-β1 and that 100% of the transformation zone biopsies expressed TGF-β1. Compared with the normal transformation zone, the SIL expressed diminished amounts of TGF-β1, thus being possibly more permissive to cancer progression in the absence of both the anti-proliferative effects and the transcriptional inhibition of E6 and E7 by TGF-β1. Inauspiciously, the SIL from several patients ceased to express detectable amounts of TGF-β1. Interestingly, a positive correlation was found between the expression of IL-10 and TGF-β1 in LG SIL of individual patients ($r^2 = 0.8$, data not shown). A positive correlation between IL-10 and TGF-β1 has also been demonstrated in a mouse tumour model system [53].

Despite the local expression of IL-12, cytokines associated with a type II response (IL-4/IL-6) and immunosuppression (IL-10)

appear to be associated with the progression of SIL [18,19]. An understanding of the interactions among multiple cytokines and other locally produced factors that contribute to the regulation of anti-tumour immunity will aid in the development of new strategies to treat SIL and cervical cancer. One potential candidate is IFN-α, a cytokine that has been shown to be effective in the treatment of HPV-associated genital warts and to have the ability to reduce the expression of IL-10 locally in basal cell carcinoma of the skin [23,54].

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