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Original Paper

Differences in Cytokine mRNA Profiles Between Premalignant and Malignant Lesions of the Uterine Cervix

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The aim of this study was to assess the expression of cytokine transcripts, reflecting the type of ongoing immune responses at the site of human papillomavirus (HPV) infection, in relation to the development of cervical neoplasia. To this end reverse transcription–polymerase chain reaction (RT–PCR) was performed for interferon (IFN)γ, interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12 (p35 and p40), and transforming growth factor (TGFβ1) in snap-frozen cervical biopsies, which were tested for the presence of high risk HPV DNA and histologically classified from normal to invasive carcinoma ($n = 40$). IFNγ, IL-10 and IL-12 (p35 and p40) transcripts were found to be expressed at significantly lower frequencies in invasive carcinoma as compared with premalignant biopsies ($P = 0.006$, $P = 0.007$ and $P = 0.002$, respectively). IFNγ and IL-10 mRNA were associated with the presence of the IL-12 p35 and p40 transcripts ($P = 0.008$ and $P < 0.00001$, respectively). These results are consistent with a locally reduced cellular (type 1) immunity correlating with HPV-induced invasive cervical carcinoma.

Key words: human papillomavirus, cervical neoplasia, cytokines, T cell, RT–PCR, IL-12, IL-10, IFNγ
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INTRODUCTION

ONCOGENIC HUMAN papillomavirus (HPV) types, of which HPV-16 and HPV-18 are the most prevalent, have been recognised as causative agents in the development of cervical cancer [1]. The failure of the host immune system to raise effective immune responses against HPV-derived antigens may contribute to cervical carcinogenesis. Indications for this can be found in studies reporting higher prevalences of HPV-induced lesions in patients with impaired cellular immune functions (reviewed in [2]).

The immune effector phase can generally be divided into two types of responses: cellular (type 1) responses and humoral (type 2) responses. T helper (Th)-1 cells and cytotoxic T lymphocytes (CTL) mediate type 1 immune responses and are responsible for the production of the characteristic type 1 cytokines interferon (IFN)γ, tumour necrosis factor (TNF)β, and interleukin (IL)-2. Type 2 responses are typically accompanied by the production of cytokines such as IL-4 and IL-5 [3]. Being the central regulators of the immune system, T helper cells are the major source of both type 1 and type 2 cytokines, but type 1 as well as type 2 cytokines can also be secreted by CD8 + CTL [3]. In addition, these cytokines can originate from other cell types, such as macrophages, granulocytes and natural killer (NK) cells [4].

The relative expression levels of type 1 and type 2 cytokines (reflecting the type of immune effector functions), are determined by the balance of certain immunomodulating cytokines, both in the initiation and the effector phase of the immune response. For instance, IFNγ and IL-4 can cross-downregulate each other's expression [4]; IL-12, which is a heterodimeric protein consisting of a 35 kDa and a 40 kDa subunit, induces the expression of type 1 cytokines and downregulates type 2 responses, while IL-10 and transforming growth factor (TGF)β can induce type 2 and suppress type 1 cytokine expression [4, 5].

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While antibody-mediated immune responses are effective in the recognition and elimination of free virions, the successful eradication of intracellular viral infections depends on cell-mediated recognition and lysis of the infected target cells [6]. Effective immunity against HPV, which are shed in the superficial layers of the dysplastic cervical epithelium, lack contact with the blood circulation and are thus without a viraemic phase, is therefore most likely to depend on cell-mediated type 1 responses [7]. The cytokines expressed in cervical biopsies are likely to reflect the ongoing type 1 or type 2 responses at the site of HPV infection, where cervical intraepithelial neoplasia (CIN) may develop. We have, therefore, explored the cytokine mRNA expression, using reverse transcription–polymerase chain reaction (RT–PCR), in 40 snap-frozen biopsies, which were also tested for the presence of high risk HPV DNA and histologically classified from normal to invasive carcinoma. Type 1 immune activity was assessed by the detection of IL-2 and IFNγ mRNA and type 2 activity by the detection of IL-4 and IL-5 mRNA. RT–PCR was also performed for IL-10, IL-12 (p35 and p40), and TGFβ1 transcripts.

MATERIALS AND METHODS

Patients, cervical biopsies and HPV typing

Snap-frozen cervical biopsy material was obtained from 24 patients with invasive cervical carcinoma (mean age 48.5 years, range 26–41 years), who attended gynaecological outpatient clinics of the Free University Hospital, the Academic Medical Centre, or the Van Leeuwenhoek Hospital, all in Amsterdam, The Netherlands. Frozen biopsy material was also collected from 16 women (mean age 31.8 years, range 26–41 years), who were referred to the oncological gynaecological out-patient clinic of the Free University Hospital after they were diagnosed with mild to moderate cervical dyskaryosis in routine smear tests. Colposcopy directed biopsies were taken and histologically classified as normal (< CIN, $n = 4$), CIN I (very mild dysplasia, $n = 3$), CIN II (mild to moderate dysplasia, $n = 3$) and CIN III (severe dysplasia, $n = 6$).

The presence of HPV DNA was determined in frozen biopsy material or in fresh cervical smear samples from the tested lesions with an HPV general primer-mediated PCR (GP-PCR) as previously described [8]. GP-PCR positive samples were used in a type specific PCR (TS-PCR), identifying the following HPV types: HPV-6, HPV-11, HPV-16, HPV-18, HPV-31 or HPV-33 [9]. If a sample was positive in the GP-PCR but negative in the TS-PCR, it was regarded as HPV-X positive.

Cells and cell lines

The HPV-18 containing human cervical cancer cell line HeLa was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and used in reconstruction experiments to assess RT–PCR detection of cytokine transcripts in T cells in a background of HPV positive keratinocytes. Positive controls for all cytokine RT–PCR reactions were included in all performed tests. For the IL-2, IFNγ, IL-4, IL-5, and IL-10 reactions, peripheral blood mononuclear cells (PBMC) from a healthy donor, stimulated for 24 h by phytohaemagglutinin (PHA) (10 μg/ml, Murex, Utrecht, The Netherlands) and the anti-CD28 monoclonal antibody 15E8 (5 μg/ml, a kind gift from Dr R. v.Lier, CLB, Amsterdam, The Netherlands) was used as a positive control. For IL-12 p35 and p40, the B-lymphoma cell line DOHH2 [10] and for TGFβ1, the bladder carcinoma cell line 5637, were used [11]. Cells were pelleted, embedded in O.C.T. compound (Miles Inc., Elkhart, Indiana, U.S.A.) and snap-frozen in liquid nitrogen. Sections were cut, RNA isolated and RT–PCR performed as described below.

RNA isolation and cDNA synthesis

In total 20 4 μm thick sections were cut from each of the snap-frozen patient tissue samples. The first and last section were subjected to acetone fixation, stained with haematoxylin and eosin, and reassessed to confirm the previously assigned histological grade. Eight sections were reserved for immunohistochemical staining. The remaining 10 sections were homogenised in 0.5 ml RNAzol (Cinna/Biotec, Houston, Texas, U.S.A.). After chloroform extraction, total RNA was precipitated in isopropanol, rinsed with 70% ethanol, lyophilised, and dissolved in 50 μl of distilled water treated with 0.1% diethylpyrocarbonate (DEPC). Subsequently cDNA synthesis was performed, using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, Wisconsin, U.S.A.) and specific antisense primers for all transcripts tested (sequences shown in Table 1). Per reaction 5 μl of RNA solution was added to 0.75 μl of antisense primer (75 pmol) and incubated at 65°C for 5 min, after which it was transferred to ice. Subsequently, 2 μl of RT buffer (500 mM Tris–HCl (pH 8.3), 600 mM KCl and 30 mM $MgCl_2$), 2 μl 100 mM dithiothreitol (DTT), 11 μl 2 mM dNTP, 0.2 μl RNA-sin (40 U/ml, Promega Biotec) and 7 U of AMV reverse transcriptase (0.2 μl, Promega Biotec) were added. The samples were incubated at 42°C for 1 h. The reaction for each sample was also performed in the absence of reverse transcriptase (− RT) to control for the subsequent amplification of any genomic DNA sequences. The obtained cDNA samples were stored at − 80°C until PCR.

PCR analysis

For PCR-mediated amplification, 5 μl of undiluted, 5×, and 25× diluted cDNA solution (in distilled water) were each added to 5 μl PCR buffer (50 mM Tris–HCl (pH 8.3), 440 mM KCl, 12 mM $MgCl_2$), 0.25 μl of the sense and 0.25 μl of the antisense (each 25 pmol) primer, 2.5 μl 2 mM dNTP, 36.8 μl distilled water and 1 U of AmpliTaq DNA polymerase (0.2 μl, Perkin–Elmer, Emeryville, California, U.S.A.) at 0°C. The reaction mixtures were amplified using an automated thermal cycler for 40 cycles (GeneAmp PCR System 9600, Perkin–Elmer, Brauchburg, New Jersey, U.S.A.), under the following conditions: denaturation at 94°C, 5 min in the first cycle, thereafter 40 sec; primer annealing at 60°C for all primer pairs, 1 min in the first cycle, thereafter 50 sec; elongation at 72°C, 1 min in the first cycle, thereafter 75 sec, except in the last cycle: 7 min. Sense (s) and antisense (as) cytokine primers containing intron-flanking sequences to distinguish the amplification of genomic sequences were selected using PCgene sequence analysis software (IntelliGenetics, Mountain View, CA), except for the TGFβ1 primers which were previously described [12]. The sequences of the used primer pairs are listed in Table 1. As RNA quality control, cDNA synthesis and subsequent PCR amplification of constitutively expressed mRNA encoding the small nuclear ribonucleoprotein specific A protein (snRNP U1A) were performed for each sample with U1A specific primers as previously described ([13], see also Table 1). Water and − RT samples were included for all reactions to check for contaminations and the co-amplification of genomic sequences, respectively.

Table 1. *Primer pairs and internal oligoprobes*

Transcript	Primer and oligoprobe sequences	Amplimer length (bp)
IL-2	s: 5′-GCAACTCCTGTCTTGCATTGC-3′ as: 5′-GTTCTGTGGCCTTCTTGGGC-3′ op: 5′-AAGAATCCCAAACTCACCAGG-3′	205
IFNγ	s: 5′-TGCAGGTCATTCAGATGTAGCGG-3′ as: 5′-ACAACCATTACTGGGATGCTCTTCG-3′ op: 5′-GGAGTCAGATGCTGTTTCAAGGTCG-3′	391
IL-4	s: 5′-ACTCTGTGCACCGAGTTGACCG-3′ as: 5′-GTCGAGCCGTTTCAGGAATCG-3′ op: 5′-GCTTGTGCCTGTGGAACTGCTGTGC-3′	197
IL-5	s: 5′-GAGCCATGAGGATGCTTCTGC-3′ as: 5′-TTGAATAGTCTTTCCACAGTACCC-3′ op: 5′-TTGACTCTCCAGTGTGCCTATTCC-3′	270
IL-10	s: 5′-GAAGATACGAAACTGAGACATCAGG-3′ as: 5′-CCCAGAGACAAGATAAATTAGAGG-3′ op: 5′-GAAAGCGTGGTCAGGCTTGGAATGG-3′	350
IL-12–p35	s: 5′-CATAACTAATGGGAGTTGCCTGGC-3′ as: 5′-AACGGTTTGGAGGGACCTCG-3′ op: 5′-CAAAACATAAAACCAGCACAGTGGACG-3′	372
IL-12–p40	s: 5′-GGCCAGTACACCTGTCACAAA-3′ as: 5′-TGATGATGTCCCTGATGAAGAAGC-3′ op: 5′-CAAAACATAAAACCAGCACAGTGGACG-3′	444
TGFβ1*	s: 5′-TCCGCAAGGACCTCGGCTGGA-3′ as: 5′-ATCATGTTGGACAGCTGCTCC-3′ op: 5′-CCTACATTTGGAGCCTGGACACGC-3′	243
U1A†	s: 5′-CAGTATGCCAAGACCGACTCAGA-3′ as: 5′-GGCCCGGCATGTGGTGCATAA-3′ op: 5′-AGAAGAGGAAGCCCAAGAGCCA-3′	221

s, sense primer; as, antisense primer; op, oligoprobe; IL, interleukin; IFN, interferon; TGFβ1, transforming growth factor β1. * [12]. † [13].

The resulting PCR products were run on 1.5% agarose gels containing ethidium bromide and visualised under ultraviolet illumination. To check the specificity of the products, they were blotted on to Nylon membranes (Qiabrane, Westburg, Germany) and hybridised with ^{32}P-γATP-labelled internal oligo probes (Table 1), overnight at 55°C. The membranes were washed three times for 20 min in 2× SSC (sodium chloride sodium citrate). Bands were subsequently visualised using a phosphor-imaging system (Molecular Dynamics, Sunnyvale, California, U.S.A.). Cytokine mRNA expression in the samples was established by a band of the correct length at either of the three tested cDNA dilutions.

Immunohistochemistry and quantitation of CD3 + cells

Snap-frozen 4 μm tissue sections (derived from the same series of sections cut for cytokine mRNA detection) were thawed, acetone fixated, pre-incubated with normal rabbit serum (1:50, 15 min, Dakopatts, Glostrup, Denmark), and then stained for CD3 (1:100, Leu-4, Becton-Dickinson, San Jose, California, U.S.A.). An irrelevant subclass matched antibody served as a negative control (MOPC 21, Organon Teknika, West Chester, Pennsylvania, U.S.A.). After incubation with a secondary biotinylated rabbit-anti-mouse antibody (1:150, 45 min, Dakopatts) and streptavidin–horseradish peroxidise complexes (1:500, 30 min, Zymed, San Francisco, California, U.S.A.), staining was visualised with amino-ethyl-carbazol (AEC). Finally, the sections were counterstained with haematoxylin and mounted. CD3 + cells were counted using a 10×10 grid overlay at a 250× magnification. The total surface area of the sections was scanned, while consistently counting the CD3 + cells positioned in the top 10 grids of the overlay. Only grids which were fully occupied with epithelium or stroma were counted. The total T cell content per section was then estimated by multiplying the cell count by 10. Counts were performed by two independent individuals. Variations in T cell counts never exceeded 10%.

Statistical analysis

Frequencies of positive cytokine mRNA expression between groups were compared using Fisher's exact test. CD3 counts were compared using the Mann–Whitney *U* test. Differences were considered significant when $P < 0.05$.

RESULTS

Cytokine mRNA expression by activated T cells in a background of HPV containing keratinocytes: sensitivity of the RT–PCR tests

The relative sensitivity of the RT–PCR methods for the detection of cytokine transcripts in activated T cells in a background of HPV positive keratinocytes was investigated. The HPV-18 containing cervical carcinoma cell line HeLa was used as a source of HPV infected and transformed keratinocytes and was found to express transcripts of TGFβ1 and IL-12 (p35 and p40), but not any of the other tested cytokines (data not shown). PHA/anti-CD28 stimulated PBMC from a healthy donor were titrated in a background of HeLa cells at ratios of 1:10, 1:10^2, 1:10^3, and 1:10^4 and without PBMC and tested for IL-2, IFNγ, IL-4, IL-5 and IL-10. For each reaction, mRNA derived from 10^4 mixed cells at the different PBMC/HeLa ratios was used. This corresponded to an absolute number of PBMC in a range from 1000 to 0 per reaction. The results are presented in Figure 1. The RT–PCR for IL-2 showed the highest sensitivity (mRNA detectable at the level of 1 PBMC in a background of 10^4 keratinocytes),

for IFNγ and IL-4 an intermediate sensitivity (at 10 PBMC in 10^4 keratinocytes), and for IL-5 and IL-10 the lowest sensitivity (at 100 PBMC in 10^4 keratinocytes).

Cytokine mRNA expression in cervical biopsies

Cytokine mRNA expression was determined in four histologically normal (< CIN), 12 dysplastic (CIN I–III), and 24 cervical carcinoma biopsies. Figure 2 shows typical data from a histologically normal biopsy, a CIN I, a CIN III, and three cervical carcinoma samples. Tables 2 and 3 summarise the scores for cytokine mRNA expression based on these results. There is an overlap in cytokine mRNA expression between groups defined by disease, but a type 1 to type 2 shift was found between normal/premalignant biopsies and carcinoma samples. All premalignant biopsies showed mRNA expression of the type 1 cytokine IFNγ (Table 2), while in 8/24 (33.3%) cervical carcinoma samples only type 2 transcripts were detected (Table 3). In no cervical sample was IL-2 mRNA detected, in contrast to the positive controls (Figure 2). Figure 3(a) summarises the frequency of cytokine mRNA detection in the different patient biopsies. IL-4 and IL-5 mRNA expression was more frequently found in carcinoma biopsies (92% and 54%) than in normal or CIN biopsies (81% and 31%, respectively), but these differences were not statistically significant. In contrast, IFNγ mRNA expression was significantly less frequent in carcinoma samples. IFNγ transcripts were detected in all 16 normal or CIN biopsies and in 15/24 carcinoma samples (63%, $P = 0.006$). Similarly, IL-10 mRNA was found in 14/16 normal or CIN samples (88%) and only in 10/24 cervical carcinoma biopsies (42%, $P = 0.007$). Also, a significant decrease in IL-12 mRNA expression rate was observed in carcinomas as compared with CIN lesions or normal cervices. Simultaneous expression of both p35 and p40 mRNA (both are required for the expression of a fully functional IL-12 heterodimer) was found in 15/16 normal or CIN biopsies (94%), but only in 10/24 carcinomas (42%, $P < 0.0025$) (Figure 3a). TGFβ1 mRNA expression was observed in virtually all biopsies, normal, premalignant and malignant. Thus, it appears that biopsies from malignant cervical lesions are less likely to have IFNγ, IL-10 and IL-12 mRNA compared with normal or premalignant biopsies.

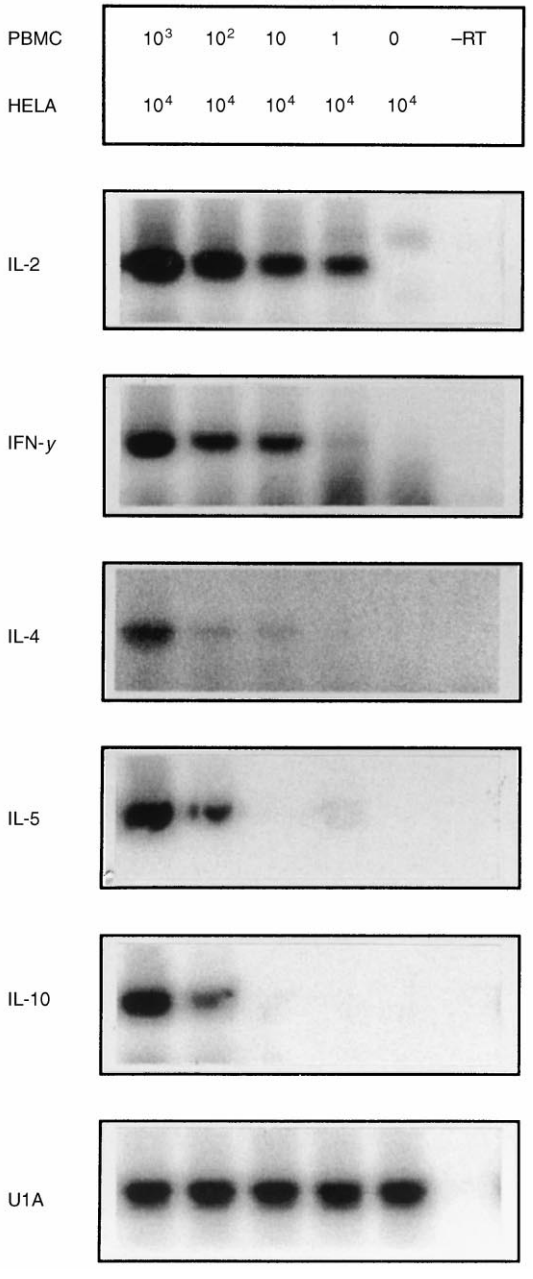

Figure 1. Reverse transcription–polymerase chain reaction (RT–PCR) assisted detection of interleukin (IL)-2, interferon (IFNγ), IL-4, IL-5, IL-10 and snRNP U1A mRNA at different ratios of PBMC (PHA/anti-CD28 stimulated) and HeLa (HPV-18+) cells. The various cell mixtures were pelleted and snap-frozen prior to mRNA extraction; all cDNA samples were used undiluted in the PCR. Also presented are results from the 10^3 PBMC:10^4 HeLa ratio without reverse transcriptase (– RT), showing that there was no co-amplification of genomic DNA sequences in the PCR. One of three representative experiments is shown.

Differences in the expression of IFNγ, IL-2, IL-4, IL-5 and IL-10 mRNA may reflect differences in the type of ongoing T cell responses. In order to rule out that any of the observed differences in expression of these cytokine mRNA transcripts might be due to a differential extent of T cell infiltration rather than to an actual change in T cell cytokine expression patterns, T cell content in the lesions was correlated to the detection of cytokine transcripts. As both type 1 and type 2 cytokines can be expressed by T helper cells and CTL, no differential CD4 and CD8 counts were performed, but rather CD3 counts. All T cells present in the used tissue sections, regardless of intraepithelial or stromal localisation, contributed to the detected cytokine mRNA signals. Therefore, the total and absolute number of T cells present in these sections was estimated. The absolute number of CD3+ cells was quantified by immunohistochemistry in 10 normal or CIN and in 10 carcinoma biopsies, which were selected to represent the range of observed cytokine expression profiles (Tables 2 and 3). Although on the whole less T cells were found in the CIN biopsies (mean 5677, range 2930–10 460) than in the carcinoma samples (mean 7828, range 2410–14 650) this difference was not significant. Moreover, the observed differential cytokine mRNA content was apparently not a reflection of a limiting quantitative T cell infiltration in a biopsy: no significant differences in T cell content between biopsies positive or negative for any type 1, type 2, or for IL-10 transcripts were found.

TGFβ1 and IL-12 mRNA in relation to type 1, type 2, or IL-10 mRNA detection

No correlation was found between the presence of TGFβ1 mRNA and the expresssion of any of the tested type 1 or type 2 cytokine transcripts, nor with IL-10. In contrast, significant differences in mRNA expression frequencies of the type-1 cytokine IFNγ and of IL-10 were found between IL-12 p35 and p40 mRNA positive biopsies and biopsies negative for

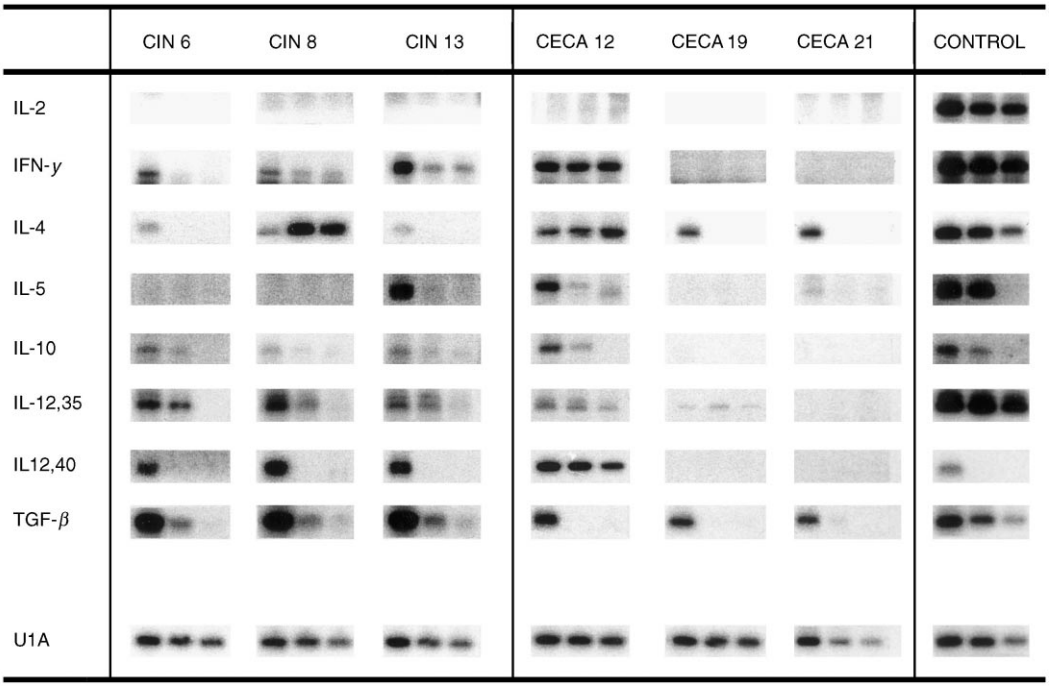

Figure 2. Cytokine mRNA expression patterns in a normal and two dysplastic cervical biopsies (CIN6: <CIN, CIN8: CIN III and CIN13: CIN I), and three cervical carcinoma (CECA 12, 19, 21) samples. The biopsy codes correspond to the order in which they are listed in Tables 2 and 3. Results are shown for interleukin (IL)-2, interferon (IFNγ), IL-4, IL-5, IL-10, IL-12 p35 and p40, transforming growth factor β1 (TGFβ1) (TGF-β), and the constitutively expressed snRNP U1A transcripts. The following positive controls (CONTROL) were used: for IL-2, IFNγ, IL-4, IL-5, IL-10 and U1A, PHA/anti-CD28 stimulated PBMC; for IL-12 p35 and p40, the B-lymphoma cell line DOHH2; for TGFβ1 the bladder carcinoma cell line 5637. Amplimers generated from cDNA in three different dilutions (1×, 5×, 25×) are shown for each sample.

Table 2. Cytokine mRNA expression in normal and dysplastic cervical biopsies analysed by reverse transcription–polymerase chain reaction (RT–PCR) (n = 16)

				Type 1		Type 2			IL-12		
Immune response	Biopsy*	CIN†	HPV‡	IL-2	IFNγ	IL-4	IL-5	IL-10	p35	p40	TGFβ1
Type 1	CIN1§	III	–	–	+	–	–	+	+	–	+
	CIN2	O	16	–	+	–	–	+	+	+	+
	CIN3	I	16	–	+	–	–	+	+	+	+
Type 1/2	CIN4§	III	–	–	+	+	–	–	+	+	+
	CIN5	III	16	–	+	+	–	–	+	+	+
	CIN6§	0	–	–	+	+	–	+	+	+	+
	CIN7§	II	–	–	+	+	–	+	+	+	+
	CIN8§	III	33	–	+	+	–	+	+	+	+
	CIN9§	O	–	–	+	+	–	+	+	+	+
	CIN10	III	16	–	+	+	–	+	+	+	+
	CIN11	II	16	–	+	+	–	+	+	+	+
	CIN12§	II	–	–	+	+	+	+	+	+	+
	CIN13§	I	33	–	+	+	+	+	+	+	+
	CIN14§	I	X	–	+	+	+	+	+	+	+
	CIN15§	III	16	–	+	+	+	+	+	+	+
	CIN16	O	16	–	+	+	+	+	+	+	+

*Ordered from more type 1-like to more type 2-like cytokine expression patterns; biopsies from patients marked with § were checked for CD3 + cell content (number of CD3 + cells per total surface of one cryostat section): biopsy no. 1, 5730; no. 4, 4980; no. 6, 2930; no. 7, 4030; no. 8, 10 460; no. 9, 6580; no. 12, 2580; no. 13, 3810; no. 14, 6580; no. 15, 9090. †Histological classification: O, no dysplasia; I, very mild dysplasia; II, mild to moderate dysplasia; III, severe dysplasia. ‡ Determined by PCR: –, HPV negative; X, positive for type other than 6, 11, 16, 18, 31 or 33. SnRNP U1A and PBMC/cell line controls were positive for all tests. CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; IL, interleukin; IFN, interferon; TGFβ1, transforming growth factor β1.

Table 3. Cytokine mRNA expression in cervical carcinoma biopsies analysed by reverse transcription–polymerase chain reaction (RT-PCR) (n = 24)

			Type 1		Type 2			IL-12		
Immune response	Biopsy*	HPV†	IL-2	IFNγ	IL-4	IL-5	IL-10	p35	p40	TGFβ1
Type 1/2	CECA1	16,18	–	+	+	–	–	–	–	+
	CECA2‡	6,16	–	+	+	–	–	–	–	+
	CECA3‡	16	–	+	+	–	–	+	+	+
	CECA4	ND	–	+	+	–	–	+	+	+
	CECA5	ND	–	+	+	–	–	+	–	+
	CECA6	16	–	+	–	+	–	–	+	+
	CECA7	16	–	+	+	–	+	+	+	+
	CECA8‡	16	–	+	+	+	–	–	+	+
	CECA9	16	–	+	+	+	–	–	–	–
	CECA10	16	–	+	+	+	+	+	+	+
	CECA11‡	16,18	–	+	+	+	+	+	+	+
	CECA12‡	16	–	+	+	+	+	+	+	+
	CECA13	X	–	+	+	+	+	–	–	–
	CECA14	X	–	+	+	+	+	+	+	+
	CECA15	ND	–	+	+	+	+	+	+	+
Type 2	CECA16‡	X	–	–	+	–	–	–	+	+
	CECA17‡	16	–	–	+	–	–	+	–	+
	CECA18‡	18	–	–	+	–	–	–	–	+
	CECA19	ND	–	–	+	–	–	+	–	+
	CECA20	16	–	–	+	+	–	+	–	+
	CECA21	ND	–	–	+	+	–	–	–	+
	CECA22‡	16	–	–	+	+	+	+	+	+
	CECA23	16	–	–	+	+	+	+	+	+
	CECA24‡	18	–	–	–	–	–	–	–	+

*Ordered from more type 1-like to more type 2-like cytokine expression patterns; biopsies from patients marked with ‡ were checked for CD3 + cell content (number of CD3 + cells per total surface of one cryostat section): biopsy no. 2, 6600; no. 3, 2410; no. 8, 10 790; no. 11, 11 560; no. 12, 14 480; no. 16, 14 650; no. 17, 2590; no. 18, 4470; no. 22, 4610; no. 24, 2780. †Determined by PCR: X, positive for type other than 6, 11, 16, 18, 31 or 33. SnRNP U1A and PBMC/cell line controls were positive for all tests. CECA, cervical carcinoma; HPV, human papillomavirus; IL, interleukin; IFN, interferon; TGFβ1, transforming growth factor β1; ND, not done.

IL-12 p35 and/or p40 transcripts (Figure 3b). Of the 25 biopsies positive for both p35 and p40 mRNA, 23 were positive for IFNγ mRNA (92%) and also 23 were positive for IL-10 mRNA (92%). In contrast, of the 15 biopsies negative for either p35 or p40 mRNA, eight were positive for IFNγ (53%, $P < 0.008$), and only two were positive for IL-10 mRNA (13%, $P < 0.00001$).

DISCUSSION

The aim of this study was to assess the expression of cytokine mRNA transcripts, reflecting the type of ongoing immune responses at the site of HPV infection, in relation to the development of cervical neoplasia. These results provide evidence for a type 1 to type 2 shift between the cytokine mRNA expression profiles of normal or premalignant CIN biopsies and biopsies from malignant cervical tumours. This pattern of type 1 versus type 2 cytokine expression may reflect an important difference in local cellular immunity in dysplastic cervical disease, which retains the ability to regress, as compared with invasive cervical carcinoma, which can no longer regress.

A reduction in the expression levels of type 1 cytokines in association with the development of HPV-induced cervical lesions has been reported in previous studies [14, 15]. Decreased IFNγ mRNA expression was found in cervical biopsies from both CIN and cervical carcinoma patients as compared with biopsies from healthy women [14] and peripheral lymphocytes from women with HPV-associated CIN lesions at multiple sites of the lower genital tract were described to show a decreased production of both IFNγ and IL-2 upon non-specific polyclonal stimulation as compared with lymphocytes from women with lesions restricted to the cervix [15]. These findings are consistent with the development and progression of premalignant cervical lesions being favoured by a bias to type 2 over type 1 responses. However, in this study the type 2 bias was apparent only in the carcinoma biopsies.

Similar changes in type 1/type 2 cytokine profiles have been described in other types of cancer without known viral aetiology (e.g. ovary, breast, colon, bladder and renal cell carcinoma, reviewed by Kiessling and colleagues [16]). Thus, the observed shifts in cytokine mRNA expression profiles may be unrelated to HPV infection, but it is impossible to draw any firm conclusions from our data in regards to this. The fact that only a proportion of the carcinoma samples and none of the high grade CIN III lesions showed a type 1 to type 2 shift indicates that this is a relatively late event in cervical neoplastic development and does not play a major role in HPV persistence and the concomitant progression of premalignant cervical lesions. Nevertheless, it cannot be excluded that the type 1/type 2 shift serves as a cofactor in late stages of tumorigenesis. Moreover, it may have important repercussions for vaccination strategies, necessitating the use of adjuvants with the ability to redirect the local cervical milieu to a cytokine composition more favourable to the activation of cellular immune effector functions.

No IL-2 mRNA was detected in any cervical biopsies. An absence of IL-2 expression either at the mRNA or at the protein level has been observed in various types of cancer [16, 17]. In a mouse model, co-expression of IL-2 with IFNγ

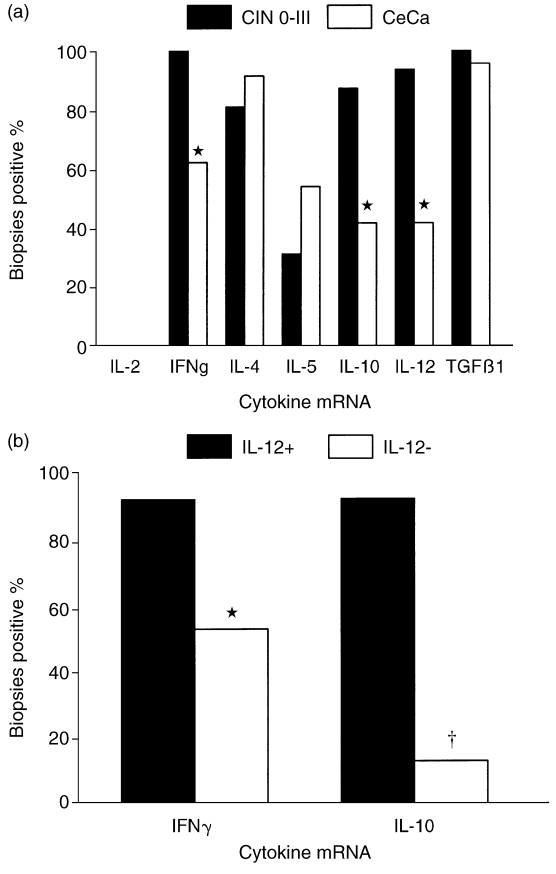

Figure 3. (a) Cytokine mRNA expression rates in normal or dysplastic (CIN, cervical intraepithelial neoplasia) biopsies (n=16; normal histology (<CIN), n=4; CIN I, n=3; CIN II, n=3; CIN III, n=6) and malignant lesions (n=24; cervical carcinoma (CeCa)). Significant differences are indicated by asterisks (P<0.01). (b) Expression frequencies of interferon (IFNγ) and interleukin (IL)-10 mRNA in the combined normal, dysplastic and malignant cervical biopsies related to the presence of IL-12 p35 and p40 transcripts (IL-12+, lesions expressing both p35 and p40 IL-12 mRNA [n=25], IL-12−; lesions lacking expression of p35 and/or p40 mRNA [n=15]). Significant differences are indicated by asterisks (* P<0.01. † P<0.00001).

has been shown to enhance synergistically tumour-specific cytotoxicity and facilitate strong memory CTL responses [18]. The absence of IL-2 mRNA in HPV-induced cervical lesions may thus explain the sporadic presence of only weak HPV-specific CTL memory responses in both CIN and cervical carcinoma patients [19]. However, previously reported HPV-16 E7 specific IL-2 production by peripheral lymphocytes, which correlated to cervical pathology [20], suggests that the observed lack of IL-2 expression in the cervix is a local phenomenon. The expression of IFNγ was found to be associated with the detection of the IL-12 p35 and p40 transcripts. This is in keeping with the important role of IL-12 in the induction of IFNγ transcription in Th1 cells, CTL and NK cells and the generation of type 1 immune responses [4]. A significantly decreased expression frequency of IL-12 mRNA was found in carcinoma biopsies as compared with normal or CIN biopsies.

Recently, IL-12 p35 and p40 mRNA expression by HPV infected keratinocytes in wart lesions was found to be associated with regression [21]. This is in agreement with our results of a preferential IL-12 p35 and p40 mRNA expression in CIN lesions, which retain the ability to regress and suggests a role for type 1 immune responses in the clearance of HPV induced lesions. It will be of particular interest to determine how the expression of IL-12 in cervical tumours will relate to their metastatic potential in clinical follow-up studies. In murine models the expression of IL-12 has been shown to have a beneficial effect on the rejection of primary tumours and the reduction of their metastatic potential [22].

The source of the IL-12 transcripts in the cervical biopsies remains to be established, but the detection of both p35 and p40 mRNA in the HPV-18 + cervical carcinoma cell line HeLa indicates that HPV infected keratinocytes, as well as antigen presenting cells (APC), are likely producers. The expression of IL-12 by keratinocytes was previously described [23] and it is conceivable that HPV induces IL-12 production by keratinocytes. Alternatively, the observed IL-12 expression may be derived from dendritic cells. In this context, it is of particular interest that a reduced production of granulocyte monocyte-colony stimulating factor (GM-CSF), IL-1β, and TNFα was previously found in cervical cancer cell lines as compared with primary cervical keratinocytes [24]. These cytokines can induce dendritic cell differentiation and concomitant IL-12 production [25]. A reduced capability of transformed keratinocytes to produce these dendritic cell maturation inducing cytokines may be related to a reduced IL-12 mRNA expression by dendritic cells in cervical carcinoma.

The type 1/type 2 dichotomy in immune responses is an operational definition but does not necessarily identify distinct cellular subsets [26]. Although IFNγ and IL-4 expression tend to be mutually exclusive at the single cell level due to the ability of each cytokine to downregulate expression of the other at the transcriptional level, this need not be the case for the other type 1 and type 2 cytokines [26]. For instance, IL-10, although often classified as a type 2 cytokine and capable of down-regulating type-1 cytokine expression, has been reported to be co-expressed with IFNγ in both CD4 + and CD8 + T cell clones at the single-cell level [27, 28].

In this study, the expression rate of IL-10 mRNA was decreased in malignant as compared with normal or premalignant biopsies, similarly to IFNγ and IL-12. Simultaneous expression of IFNγ and IL-10 has previously been reported both in solid tumours and certain infectious diseases [16]. Moreover, both IFNγ and IL-10 expression by T cells can be upregulated by IL-12 [27, 28]. This IL-12 mediated upregulation of IL-10 occurs at the transcriptional level and is specific to T cells; IL-10 production by macrophages cannot be upregulated by IL-12 [29]. The close association observed between IL-12 and IL-10 mRNA detection (which is even stronger than the association between IL-12 and IFNγ mRNA [Figure 3b]) thus strongly suggests that the IL-10 mRNA detected in the cervical biopsies is produced by T cells. We hypothesise that both IFNγ and IL-10 transcripts present in cervical dysplastic lesions are expressed by type 1 T cells and that this expression is regulated by IL-12. Production of IL-10 by such T cells may serve a negative feedback function after protracted periods of antigenic stimulation (as may be the case in cervical lesions induced by persistent HPV infections) by downregulating IL-12 expression, thus preventing uncontrolled local inflammatory responses [29]. In keeping with this hypothesis, production of IL-10 by *in vitro* primed human Th1 cells was recently reported after several rounds of restimulation in the presence of IL-12 [30]. Alternatively, both IL-12 and IL-10 may

be produced by HPV infected keratinocytes and may be simultaneously downregulated in cervical carcinomas. Immunohistochemical or RNA *in situ* hybridisation studies will need to be performed to clarify these matters. In any case, the observed co-expression of IL-10 and IL-12 in this study may be a strictly local phenomenon as IL-10 and IL-12 were recently reported to be present at, respectively, higher and lower concentrations in whole blood culture supernatants of patients with cervical dysplasia as compared with culture supernatants from normal controls [31].

Although TGFβ1 was previously reported to upregulate IL-10 expression [16, 32], we found no correlation between TGFβ1 and IL-10 mRNA expression. However, TGFβ1 mRNA was found in virtually all samples, so that correlations would have called for an accurate quantitation of the cytokine transcripts. Moreover, TGFβ1 is produced as an inactive procytokine which needs to be cleaved before it can exert its regulatory function [33]. It may, therefore, be more appropriate to study TGFβ1 (active) protein expression by immunohistochemistry in future studies to clarify this point further.

In conclusion, cervical carcinoma biopsies express IL-12 mRNA less frequently than premalignant lesions, which may reflect a decrease in local type 1 T cell mediated immunity. This observation is consistent with a role for IL-12 and type 1 immune responses in the control of cervical carcinoma development and favours the view that the inclusion of IL-12 as adjuvant may enhance the efficacy of therapeutic vaccination protocols with HPV-derived antigens.

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