PERGAMON

European Journal of Cancer 39 (2003) 595–603

European Journal of Cancer

www.ejconline.com

Identification and characterisation of a group of cervical carcinoma patients with profound downregulation of intratumoral Type 1 (IFNγ) and Type 2 (IL-4) cytokine mRNA expression

A. Gey[a], P. Kumari[b], A. Sambandam[b], F. Lecuru[c], L. Cassard[a], C. Badoual[d], C. Fridman[a], B. Nagarajan[b], W.H. Fridman[a], E. Tartour[a,*]

[a]*Unité d'immunologie biologique, Hopital Européen Georges Pompidou, INSERM U255, Université Pierre et Marie Curie, 20 Rue Leblanc 75908, Paris Cedex 15, France*

[b]*Unité d'anatomie pathologique, Hopital Européen Georges Pompidou, Paris, France*

[c]*Service de gynécologie chirurgicale, Hopital Européen Georges Pompidou, Paris, France*

[d]*Department of Microbiology and Tumor Biochemistry Cancer Institute Chennai, India*

Received 8 April 2002; received in revised form 29 August 2002; accepted 23 October 2002

Abstract

Type 1 cytokines, such as interferon gamma (IFNγ) and interleukin-2 (IL-2), increase T cell-mediated immune responses and are considered to be beneficial for antitumour immunity. Type 2 cytokines, such as IL-4, IL-5, and IL-10, inhibit Type 1 responses and promote humoral responses. We have previously reported an association between low intratumoral *IFNγ* mRNA levels and poor clinical outcome in patients with invasive cervical carcinoma. In this study, by using quantitative polymerase chain reaction (PCR), we identified a group of cervical carcinoma patients with undetectable intratumoral T cell-derived cytokine mRNAs, as *IFNγ*, *IL-4* and *IL-17* expression could not be detected in 5, 25 and 8 of the 52 biopsies analysed, respectively. Global downregulation of Type 1 and Type 2 cytokines was observed in a subgroup of patients who more frequently presented advanced stage tumours. Biopsies of patients with no *IFNγ* gene expression did not appear to be less infiltrated by T cells than control biopsies with measurable *IFNγ* gene expression. These results clearly demonstrate that, in some clinical situations, the decrease in intratumoral Type 1 cytokines is not associated with a Type 2 polarisation, but rather reflects global deactivation of T cells at the tumour site. These data provide support for immunotherapy protocols designed to reverse the anergic state of T cells in cancer.

1. Introduction

Cervical cancer is the third most frequent female cancer worldwide with 400 000 new cases diagnosed each year, resulting in approximately 200 000 deaths per year with the greatest burden of disease in developing countries [1].

Infection with certain human papillomaviruses (HPV) has been identified as a major risk for the development of cervical neoplasia and cervical cancer [2]. Virtually all cervical cancers contain the genes of high-risk HPV (most commonly, types 16,18, 31 and 45). HPV early nuclear protein E6 and E7 expression is necessary and sufficient for transformation of primary keratinocytes [3]. The E7 protein is required for maintenance of the proliferative state of HPV-infected cells [4].

Different arguments suggest that cell-mediated immune responses are essential to control the growth of HPV-associated tumours: (i) The prevalence of HPV-related diseases (infection and neoplasm) is increased in transplanted patients and HIV-infected patients [5,6]. (ii) The growth of HPV-associated tumours has been prevented by induction of anti-HPV effector T cells in various animal models, as immunisation of rodents with E7 recombinant vectors, chimeric E7 virus-like particles, dendritic cells pulsed with E7 protein, purified E7 protein or derived peptides mixed with adjuvants yielded protection against E7-expressing tumours [7–11]. (iii) In man, the presence of T lymphocytes in spontaneously regressing HPV-related papillomas [12] and HPV-specific T cells in tumour infiltrating lymphocytes derived from cervical carcinoma patients have also been docu-

* Corresponding author. Tel.: +33-1-5609-3942; fax: +33-1-5609-2080.

E-mail address: eric.tartour@egp.ap-hop-paris.fr (E. Tartour).

0959-8049/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0959-8049(02)00839-0

mented [13]. Recently, a correlation was demonstrated between cellular immunity to HPV-16 E7 measured by skin test and clinical and cytological resolution of HPV-induced Cervical Intraepithelial Neoplasia (CIN) [14].

Cytokines are molecules initially identified as agents allowing activation and differentiation of immunocompetent cells. They also directly influence carcinogenesis and metastasis by modifying the tumour phenotype [15].

Cytokines produced during antigenic stimulation may polarise the immune response. A Type 1 response involving interferon gamma (IFNγ), interleukin-2 (IL-2), and IL-12 has been shown to increase T cell-mediated immunity and is considered to be beneficial for antitumour immunity. A type 2 response is characterised by production of IL-4, IL-5, IL-6, IL-9 and IL-10, which promotes humoral immunity and inhibits Type 1 cytokine production and cytotoxic T lymphocyte development [16]. IL-17 is produced by helper CD4-TH0 T cells which are considered to be precursor cells sensitive to environmental signals for their differentiation and maturation [16].

Various data suggest progressive downregulation of Type 1 cytokines (IFNγ, IL-2) associated with an increase of Type 2 cytokines in cancer [17–19]. This type 1-to-type 2 cytokine shift may reflect a reduced protective cell-mediated immunity against tumours [20]. Our group has previously reported an association between low intratumoral IFNγ mRNA levels and poor clinical outcome in patients with primary invasive cervical carcinoma [21]. Other groups have shown a significant decrease of *IFNγ* gene expression during progression of cervical lesions from CIN to invasive cancer [22–25].

Independent studies have also highlighted the anergic state of T cells in some cancer patients [26,27].

In this study, we investigated whether this downregulation of Type 1 cytokines in cervical carcinomas is associated with a Type 2 polarisation of the immune response or reflects global deactivation of intratumoral T cells.

For this purpose, we used quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to measure the expression of Type 1 (*IFNγ*), Type 2 (*IL-4*) and TH0 (*IL-17*) cytokine mRNA typically transcribed in activated T cells in 52 biopsies derived from patients with primary invasive cervical carcinomas.

2. Patients and methods

2.1. Patients and tissues

Biopsy specimens were obtained from primary cervical lesions of 52 patients with invasive squamous cell carcinoma prior to any treatment. Tissues were divided into two equal parts: one portion was fixed in formalin for histological analysis, the other was snap frozen in liquid nitrogen and stored at −70 °C for RNA extraction. This protocol was approved by a regional ethics committee.

Clinical staging was performed according to the classification of the International Federation of Gynecology and Obstetrics (FIGO) [28]. Stages Ia and Ib were grouped into class I (16 cases), stages IIa and IIb into class II (21 cases) and stages IIIa and IIIb into class III (15 cases). HPV typing was not performed on these samples.

2.2. Preparation of mRNA and cDNA synthesis

Total cellular RNAs were extracted using the RNA Plus Kit (Quantum-Appligene, 67402 Ilkirch, France). Five micrograms of total cellular RNAs were reverse transcribed with random hexadeoxynucleotide primers using the first strand cDNA synthesis kit (Roche Molecular Biochemicals, 38242 Meylan, France) according to the manufacturer's protocol.

2.3. Quantification of mRNA

Quantification was performed by real-time RT-PCR. The method is based on direct detection of the amplified product by the release of a fluorescent reporter dye from a specific fluorescent labelled probe during PCR due to the 5′-3′ nuclease activity of the Taq DNA polymerase enzyme [29].

All PCR reactions for cytokine mRNA quantification were performed on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, 91961 Courtaboeuf, France). PCR primers and TaqMan Fluorogenic DNA probes for *β actin*, *IFNγ*, *IL-4*, *IL-17* were purchased from Applied Biosystem.

For each PCR run, 45 μl of a ready to use master mix (1× TaqMan buffer, 5 mM $MgCl_2$, 200 μM deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanidine triphosphate (dGTP), and 400 μM deoxyuridine triphosphate (dUTP), 300 nM each primer, 150 nM labelled probe, 1.25 units of AmpliTaq Gold DNA polymerase, 2.5 units of AmpErase uracil *N*-glycosylase (Applied Biosystems) were mixed with 5 μl of cDNA which corresponds to 0.5 μg of the RNA transcribed. The tubes were placed in the ABI prism System programmed as follows: one 2-min cycle at 50 °C (Uracyl *N*-glycosylase activation); one 10-min cycle at 95 °C (activation of Gold ampliTaq, and inactivation of UNG); followed by 45 sequential cycles, each comprising heating to 95 °C for 15 s and a 30 s annealing/extension step at 60 °C. Duplicate reaction tubes were set up for each sample and the mean values were calculated. All samples with a coefficient of variation $>5\%$ were retested.

The PCR cycle at which the amount of amplified target generated a detectable specific fluorescent signal that reaches a fixed threshold was defined as C_t. The larger the starting copy number of cDNA, the lower the C_t values. *β actin* was also quantified for each sample. The C_t for each parameter tested was therefore normalised by subtracting the C_t of its corresponding *β actin*. Table 1 provides an example of cytokine quantification and demonstrates the linearity of the technique.

The comparative C_t method was used to determine relative quantification of gene expression for the various samples: relative gene expression between sample A and sample B is given by the formula: $2^{\Delta Ct = CtA - CtB}$. For example, a ΔC_T of 3.32 between sample A and sample B corresponded to a $2^{3.32} = 10$-fold higher mRNA concentration of B compared with A.

The limit of linearity of this technique and background problems occur for $C_t > 37$. Above this threshold, samples were considered to be negative.

Negative controls lacking template cDNA were always included in each experiment.

2.4. Immunocytochemistry

Immunohistochemical staining was performed using the universal rabbit-mouse labelled streptavidin-biotin method (Dako LSAB kit; Dakp Corp., Santa Barbara, CA, USA). Enzymatic activity of alkaline phosphatase-conjugated streptavidin was revealed with Fas Red reagent (Dako) associated with 1 mM of levamisole, a known inhibitor of endogenous alkaline phosphatase.

T lymphocytes were characterised by antibodies to T cells (anti-CD3 rabbit polyclonal antibody at a 1:100 phosphate-buffered saline (PBS) dilution; Dako Corp). Pre-immune rabbit serum was used as isotype control antibodies.

Table 1
Quantification of *IL-17* and *β actin* mRNA from various concentrations of activated T cells

	Real-time PCR		
	β actin C_T value	*IL-17* C_T value	(*IL-17-β actin*) $\Delta\Delta C_T$
Activated T cells 2×10^5	24.94	31.39	6.45
Activated T cells 10^6	21.59	28.03	6.44
Activated T cells 5×10^6	19.63	25.66	6.03

Normal T cells were activated with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) and ionomycin (1 mM). RNA extraction and cDNA synthesis were performed 8 h after stimulation. Quantification of *IL-17* and *β actin* was performed as indicated in the Patients and methods section. PCR, polymerase chain reaction.

We evaluated cell infiltration by counting random high-power fields with an American Optical microscope using a 45× objective with a 0.47 mm diameter field.

Semi-quantitative measurement of T cell infiltration was performed according to the following criteria: 1–10 T cells/field = +; 11–50 T cells/field = + +; more than 50 T cells/field = + + +.

2.5. Statistical analysis

Relationships between cytokine gene expression and clinical stage groups were analysed by the two-sided χ^2 test with Yates correction when necessary.

3. Results

3.1. Heterogeneity of cytokine mRNA expression in invasive cervical carcinoma patients

In a first series of experiments, we measured *IFNγ* (a Type 1 cytokine), *IL-4* (a Type 2 cytokine) and *IL-17* (a TH0 cytokine) mRNA concentrations in 52 invasive cervical biopsy specimens. A marked heterogeneity in the level of intratumoral *IFNγ*, *IL-4* and *IL-17* mRNA was observed (Fig. 1). Relative gene expression between sample A and sample B was given by the formula: $2^{\Delta Ct = CtA - CtB}$. An arbitrary value of 1 was assigned to samples with the highest $C_{T(\text{cytokine}-\beta\ \text{actin})}$ value that means with the lowest mRNA concentrations detectable.

Among invasive cervical carcinoma patients with detectable cytokine mRNA expression, relative values between the various samples ranged from 1 to 826 for *IFNγ*, 1 to 103 for *IL-4* and 1 to 522 for *IL-17*. All our results were normalised to *β actin* gene expression to standardise the RNA extraction and cDNA synthesis steps between the various samples and for control of sample integrity.

The median value for $C_{t(\text{cytokine}-\beta\ \text{actin})}$ was 14.29 for *IFNγ*, 19.88 for *IL-4* and 15.55 for *IL-17* (Table 2).

Table 2
Main characteristics of the mRNA expression for *IFNγ*, *IL-4* and *IL-17* in biopsies derived from invasive cervical carcinomas

	IFNγ	*IL-4*	*IL-17*
Max (C_T)	19.35	21.59	20.60
Min (C_T)	9.66	14.89	11.57
Median (C_T)	14.29	19.88	15.55
Standard deviation	1.7	1.5	2.3
Number of cases with undetectable values	5/52	25/52	8/52

All of the C_T are normalised to *β actin* gene expression.

3.2. Identification of a group of cervical carcinoma patients with undetectable intratumoral cytokine mRNA expression

This real-time quantitative PCR also identified a group of patients with undetectable cytokine mRNA expression (Fig. 1), as no *IFNγ*, *IL-4* and *IL-17* expression was detected in 5, 25 and 8 of the 52 biopsies analysed, respectively (Fig. 1 and Table 2). A relationship was observed between the absence of expression of these cytokines, as none of the five biopsies with no *IFNγ* expression expressed *IL-4* and *IL-17* was detected in only 1 case. Similarly, 7 of the 8 biopsies that did not express *IL-17* were found to be negative for *IL-4* expression (data not shown). A bias due to the quality of mRNA tested was excluded, as the cervical tissue samples were considered to be eligible for study when the *β actin* C_T value was <25 suggesting an appropriate starting amount and quality of total RNA.

Since T lymphocytes are the major cells producing these cytokines, we investigated a possible correlation between the absence of T cell-derived cytokine mRNA expression and absence of infiltration of CD3-positive T cells in the tumour. As slides were not available for all cases, we limited this study to the group of patients with undetectable *IFNγ* mRNA expression for whom immunocytochemistry could be performed.

Table 3 clearly shows that the absence of *IFNγ* gene expression was not due to defective recruitment of T cells inside the tumour (Fig. 2). Furthermore, no correlation was observed between intratumoral *IFNγ* mRNA concentrations and the levels of T cell infiltration. Because of insufficient materials, a detailed phenotypic analysis of the T cells from this group of patients could not be performed. However, in each of the 3 cases studied with undetectable *IFNγ* levels, we could show that the infiltrating T cells did not express the CD25 activation marker (data not shown).

3.3. Correlation between cytokine mRNA expression and clinical stage

No clear correlation was observed between the levels of *IFNγ*, *IL-4* or *IL-17* mRNA expression and the clinical stage of patients with detectable cytokine mRNA expression (data not shown). However, patients with undetectable cytokine mRNA expression more often presented with advanced stage cervical carcinoma (stages II and III) rather than localised and good prognosis stage I cervical carcinomas (Fig. 3). Patients with no *IL-4* gene expression were significantly more frequent in the stages II and III groups ($P=0.01$). All patients with no *IFNγ* or *IL-17* mRNA presented stages II or III invasive cervical carcinoma (Fig. 3). Absence of activated T cell derived cytokine was therefore observed less frequently in stage I patients than in patients with more advanced stages. These results cannot be explained by a patient recruitment bias, as the number of patients included in each stage (stage I: $n=16$; stage II: $n=21$; stage III: $n=15$) was fairly homogeneous.

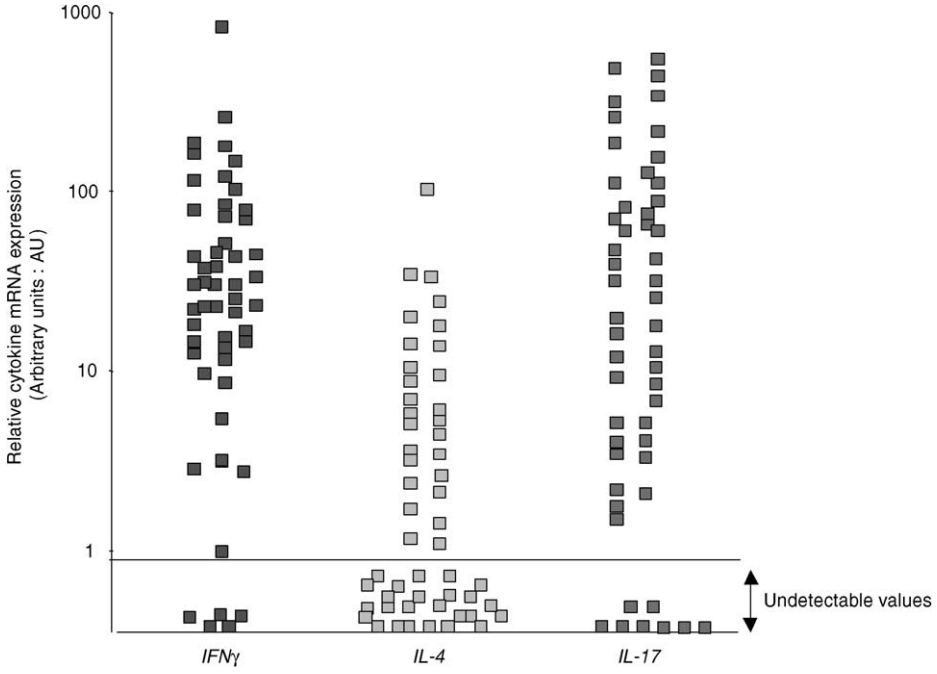

Fig. 1. Intratumoral heterogeneity of cytokine mRNA expression for *IFNγ*, *IL-4* and *IL-17* in biopsies derived from invasive cervical carcinoma patients. Quantification of mRNA was performed using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Relative mRNA values from one patient to another are given by the formula $2^{\Delta Ct}$. An arbitrary value of 1 was assigned to the sample with the highest detectable C_T. All our results were normalised to *β actin* gene expression.

4. Discussion

We have identified and characterised a group of cervical carcinoma patients with undetectable intratumoral T cell derived cytokine mRNAs, such as *IFNγ*, *IL-4* and *IL-17*.

No *IFNγ* was detected in 5/52 patients (9.6%) and the lack of expression appeared to be more frequent in advanced stage II and III tumors than in localised stage I tumours, although the difference was not statistically significant. This result is reminiscent of a study in which we demonstrated a correlation between low levels of intratumoral *IFNγ* mRNA and a subgroup of poor prognosis cervical carcinoma patients [21]. In a previous study, in which we analysed cytokine mRNA expression in biopsies derived from normal cervix or CIN, we did not identify any group of patients with undetectable levels of *IFNγ* [30].

Various studies have also demonstrated a decrease in the expression of *IFNγ* and *IL-12*, an *IFNγ* inducer, in invasive carcinoma compared with premalignant biopsies or normal cervix [23–25]. It was recently shown that HPV16 E6 and E7 proteins inhibited local IL-18-induced IFNγ production in HPV lesions via inhibition of IL-18 binding to its α chain receptor [31]. A defect in IFNγ expression at the tumour site may therefore promote tumour progression, as, *in vitro*, IFNγ inhibits expression of HPV Type 16 and 18 genes in immortalised cell lines and inhibits the growth of most cervical carcinoma cell lines [32]. A role for IFNγ for tumour rejection has also been demonstrated in many murine tumour models [33–34].

The absence of *IFNγ* mRNA expression cannot be explained by a defect of T cell recruitment inside the tumour, as biopsies from patients with no *IFNγ* expression did not appear to show less T cell infiltration than control biopsies with measurable IFNγ gene expression.

The group of patients with no detectable *IFNγ* expression also did not express *IL-4* mRNA. In our series of patients, *IL-4* mRNA could not be detected in more than 48% (25/52) of cervical carcinoma biopsies tested, especially in the advanced stage cancers. This result may seem contradictory to the accumulating data showing a switch from Type 1 to Type 2 cytokines in cancer [35]. In fact, only one study has reported an increase in intratumoral *IL-4* mRNA expression in cervical cancer compared with dysplasia or normal cervix [36]. The presence of IL-4 was correlated with eosinophil infiltration [37]. However, the levels of IL-4 expression in various stages of tumours were not determined.

Table 3
Analysis of T cell infiltration in invasive cervical carcinoma patients with undetectable (group I) or detectable (group II) *IFNγ* gene expression

Patients	Intratumoral *IFNγ* mRNA concentration (C_T)	Levels of T cell infiltration
Group I		
1	Undetectable	+ + +
2	Undetectable	+ +
3	Undetectable	+ +
4	Undetectable	+ + +
5	Undetectable	+ + +
Group II		
6	14.11	+ +
7	14.42	+ +
8	14.37	+ + +
9	4.47	+ +
10	15.26	+ + +
11	16.92	+
12	13.34	+ +

Semiquantitative determination of T cell infiltration was performed by immunocytochemistry according to the following criteria: 1–10 T cells/field (0.47 mm in diameter with a 45× objective) = +; 11–50 T cells/field = + +; more than 50 T cells/field = + + +.

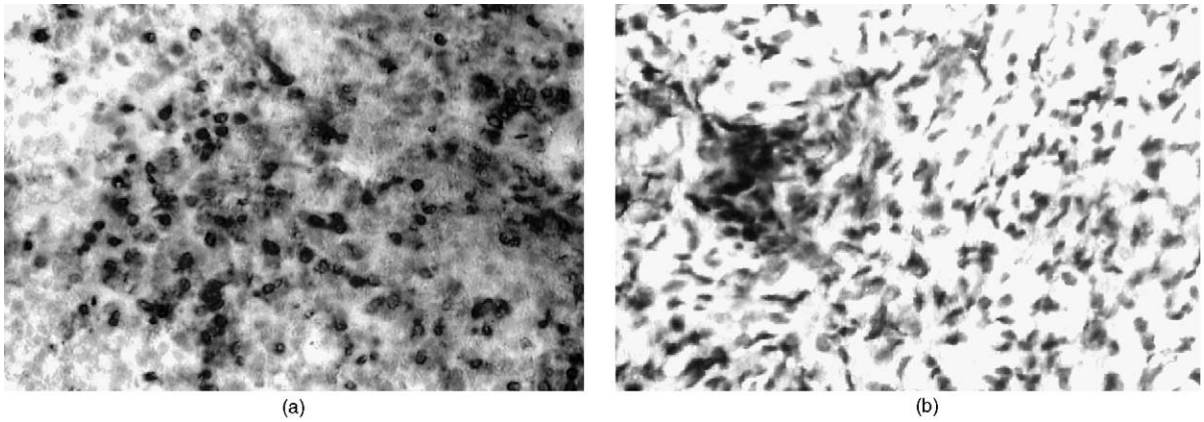

Fig. 2. T-lymphocyte recruitment in biopsies derived from invasive cervical carcinoma patients. Sections of biopsies derived from invasive cervical carcinoma patients were stained with anti-CD3 antibodies (a) or isotype control antibodies (b). Cell infiltration was assessed by counting random high-power fields with an American Optical microscope using a 45× objective with a 0.47-mm diameter field.

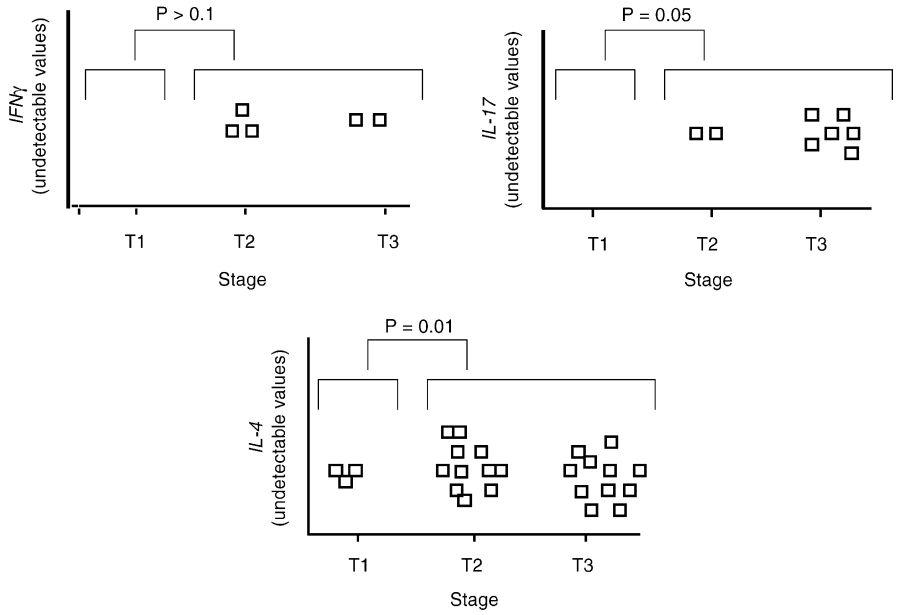

Fig. 3. Correlation between undetectable intratumoral cytokine mRNA values and clinical stage of patients with invasive cervical carcinomas. Each square corresponds to patient with undetectable values of the cytokine. All cervical tissue samples with undetectable cytokine mRNA expression ($C_T > 37$) exhibited a *β-actin* C_T value < 25.

In most other cancers, including ovarian cancer, renal cell carcinoma or colorectal carcinoma, IL-4 expression is also detected in a very few biopsies, as in the present study [38–41].

Although it has been shown that IL-4, produced by T-helper CD4-T cells, has adverse effects on the cell-mediated immune response, many studies have clearly demonstrated a major role of IL-4 to elicit CTL and induce antitumour immunity [42–45]. In man, clinical response to immunotherapy is associated with intratumoral induction of *IL-4* mRNA [46]. Downregulation of IL-4 in advanced stage cancers may therefore be considered to be a tumour escape mechanism.

Except in the case of skin cancers and certain haematological malignancies which constitutionally produce IL-4 [18,19,47], the switch from Type 1 to Type 2 cytokines described in many human cancers mainly depends on upregulation of IL-6 and IL-10 and not IL-4 [15,30,38,48,49]. In these models, cells derived from the monocyte-macrophage lineage and tumour cells have been reported to represent the main source of IL-6 and IL-10.

When tumour-derived T cells were shown to produce IL-10, they did not secrete IL-4. These cells should therefore not be called Type 2 T cells, but should be considered to be immunoregulatory T cells belonging to the Tr1 cell population [50].

IL-17, a TH0 cytokine, mainly released by CD4 T cells, was not expressed in 15% of biopsies derived from cervical carcinoma patients (Fig. 1). The absence of *IL-17* mRNA expression was only observed in stage II and III cancers and, in most cases, was associated with an absence of *IL-4* and *IFNγ* expression. We recently showed that IL-17 inhibited tumour cell growth in immunocompetent mice via a T cell-dependent mechanism and may therefore be beneficial for the host antitumour immune response [51].

The identification of a group of cervical carcinoma patients who did not express intratumoral activated T cell-derived cytokines despite intense T cell infiltration of their tumours strongly suggests that these intratumoral T cells were in a resting or anergic state. These results are supported by another study, which showed that freshly isolated tumour-infiltrating lymphocytes derived from cervical carcinoma did not exhibit any antitumour cytotoxicity [52]. In addition, a T cell response against HPV E7 derived peptides was weaker and detected less often in cervical cancer patients than in patients with CIN, which also provides further evidence for a functional defect of cervical carcinoma-derived T cells [53,54]. In other cancers, different groups have reported an anergic state of intratumoral T lymphocytes, sometimes associated with a quantitative or qualitative defect in T-cell receptor (TCR)-associated signal transduction molecules, particularly the zeta chain [55–58]. Kono and colleagues demonstrated a marked decrease in the expression of the signal-transducing CD3 zeta chain of T lymphocytes derived from patients with cervical cancer [59].

The mechanisms responsible for the global downregulation of Type 1, Type 2 and TH0 cytokines in the group of cervical carcinoma patients have yet to be determined.

IL-10 and transforming growth factor β (TGFβ) are two immunoregulatory cytokines that are able to downregulate both Type 1 and Type 2 cytokines [60,61].

Cervical cancer cells, but not normal cervical epithelial cells, have been reported to produce these two cytokines [62]. IL-10 and TGFβ expression also increase during the progression from carcinoma *in situ* to invasive cancer [62]. The genotype predisposing to the production of high levels of IL-10 is more often observed in cervical cancer patients than in healthy women [63].

In conclusion, we have characterised a group of cervical carcinoma patients with profound intratumoral downregulation of Type 1, Type 2 and TH0 activated T cell derived cytokines in the absence of a defect of T cell recruitment at the tumour site. These results strongly suggest that, in this clinical situation, the decrease of Type 1 cytokines was not associated with a Type 2 polarisation of T cells, but rather reflects global deactivation of these cells. These findings, together with data showing that the presence of intratumoral cytokine-producing T cells is associated with a better prognosis [21,46,64,65], provide support for immunotherapy protocols designed to stimulate T cells by *ex vivo* activation [66] or that modify their phenotype to make them more resistant to immunosuppressive factors [67].

Acknowledgements

This work was supported by grants from: Indo-French Center for the Promotion of Advanced Research (IFCPAR) Association pour la Recherche sur le Cancer (No. 970RC24), INSERM and Université Pierre and Marie Curie.

References

1. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers. *Int J Cancer* 1990, **1999**, 83 18-29.
2. Zur Hausen H. Papillomavirus infections—a major cause of human cancers. *Biochim Biophys Acta* 1996, **1288**, F55–F78.
3. Munger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J* 1989, **8**, 4099–4105.
4. Crook T, Morgenstern JP, Crawford L, Banks L. Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-ras. *EMBO J* 1989, **8**, 513–519.
5. Wu TC, Kurman RJ. Analysis of cytokine profiles in patients with human papillomavirus-associated neoplasms. *J Natl Cancer Inst* 1997, **89**, 185–187.
6. Laga M, Icenogle JP, Marsella R, *et al*. Genital papillomavirus infection and cervical dysplasia–opportunistic complications of HIV infection. *Int J Cancer* 1992, **50**, 45–48.
7. Meneguzzi G, Cerni C, Kieny MP, Lathe R. Immunization against human papillomavirus type 16 tumor cells with recombinant vaccinia viruses expressing E6 and E7. *Virology* 1991, **181**, 62–69.
8. Greenstone HL, Nieland JD, de Visser KE, *et al*. Chimeric papillomavirus virus-like particles elicit antitumor immunity against the E7 oncoprotein in an HPV16 tumor model. *Proc Natl Acad Sci USA* 1998, **95**, 1800–1805.
9. De Bruijn ML, Schuurhuis DH, Vierboom MP, *et al*. Immunization with human papillomavirus type 16 (HPV16) oncoprotein-loaded dendritic cells as well as protein in adjuvant induces MHC class I-restricted protection to HPV16-induced tumor cells. *Cancer Res* 1998, **58**, 724–731.
10. Feltkamp MC, Smits HL, Vierboom MP, *et al*. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol* 1993, **23**, 2242–2249.
11. Gerard CM, Baudson N, Kraemer K, *et al*. Therapeutic potential of protein and adjuvant vaccinations on tumour growth. *Vaccine* 2001, **19**, 2583–2589.
12. Coleman N, Birley HD, Renton AM, *et al*. Immunological events in regressing genital warts. *Am J Clin Pathol* 1994, **102**, 768–774.
13. Evans EM, Man S, Evans AS, Borysiewicz LK. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. *Cancer Res* 1997, **57**, 2943–2950.
14. Hopfl R, Heim K, Christensen N, *et al*. Spontaneous regression of CIN and delayed-type hypersensitivity to HPV-16 oncoprotein E7. *Lancet* 2000, **356**, 1985–1986.
15. Tartour E, Fridman WH. Cytokines and cancer. *Int Rev Immunol* 1998, **16**, 683–704.
16. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996, **17**, 138–146.
17. Ghosh P, Komschlies KL, Cippitelli M, *et al*. Gradual loss of T-helper 1 populations in spleen of mice during progressive tumor growth. *J Natl Cancer Inst* 1995, **87**, 1478–1483.
18. Saed G, Fivenson DP, Naidu Y, Nickoloff BJ. Mycosis fungoides exhibits a Th1-type cell-mediated cytokine profile whereas Sezary syndrome expresses a Th2-type profile. *J Invest Dermatol* 1994, **103**, 29–33.
19. Yamamura M, Modlin RL, Ohmen JD, Moy RL. Local expression of anti-inflammatory cytokines in cancer. *J Clin Invest* 1993, **91**, 1005–1010.
20. Clerici M, Shearer GM, Clerici E. The tumor enhancement phenomenon: reinterpretation from a Th1/Th2 perspective. *J Natl Cancer Inst* 1996, **88**, 461–462.
21. Tartour E, Gey A, Sastre-Garau X, Lombard-Surin I, Mosseri V, Fridman WH. Prognostic value of intratumoral interferon gamma messenger RNA expression in invasive cervical carcinomas. *J Natl Cancer Inst* 1998, **90**, 287–294.
22. Mori H, Hanabayashi T, Yamada Y, Tamaya T. Decrease in interferon-gamma production by peripheral blood mononuclear cells in patients with uterine cervical cancer. *J Clin Immunol* 1990, **10**, 45–51.
23. de Gruijl TD, Bontkes HJ, van den Muysenberg AJ, *et al*. Differences in cytokine mRNA profiles between premalignant and malignant lesions of the uterine cervix. *Eur J Cancer* 1999, **35**, 490–497.
24. El-Sherif AM, Seth R, Tighe PJ, Jenkins D. Quantitative analysis of IL-10 and IFN-gamma mRNA levels in normal cervix and human papillomavirus type 16 associated cervical precancer. *J Pathol* 2001, **195**, 179–185.
25. Pao CC, Lin CY, Yao DS, Tseng CJ. Differential expression of cytokine genes in cervical cancer tissues. *Biochem Biophys Res Commun* 1995, **214**, 1146–1151.
26. Tartour E, Latour S, Mathiot C, *et al*. Variable expression of CD3-zeta chain in tumor-infiltrating lymphocytes (TIL) derived from renal-cell carcinoma: relationship with TIL phenotype and function. *Int J Cancer* 1995, **63**, 205–212.
27. Lee PP, Yee C, Savage PA, *et al*. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med* 1999, **5**, 677–685.

28. Petterson, F., ed. Annual report on the results of treatment in gynecological cancer, vol. 20. Stockholm, International Federation of Gynecology and Obstetrics, 1988, p 30–31.
29. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996, **10**, 986–994.
30. Tartour E, Gey A, Sastre-Garau X, Pannetier C, Mosseri V, Kourilsky P, Fridman WH. Analysis of interleukin 6 gene expression in cervical neoplasia using a quantitative polymerase chain reaction assay: evidence for enhanced interleukin 6 gene expression in invasive carcinoma. *Cancer Res* 1994, **54**, 6243–6248.
31. Lee SJ, Cho YS, Cho MC, *et al*. Both E6 and E7 oncoproteins of human papillomavirus 16 inhibit IL-18-induced IFN-gamma production in human peripheral blood mononuclear and NK cells. *J Immunol* 2001, **167**, 497–504.
32. Woodworth CD, Lichti U, Simpson S, Evans CH, DiPaolo JA. Leukoregulin and gamma-interferon inhibit human papillomavirus type 16 gene transcription in human papillomavirus-immortalized human cervical cells. *Cancer Res* 1992, **52**, 456–463.
33. Qin Z, Blankenstein T. CD4+ T cell–mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity* 2000, **12**, 677–686.
34. Shankaran V, Ikeda H, Bruce AT, *et al*. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 2001, **410**, 1107–1111.
35. Clerici M, Shearer GM, Clerici E. Cytokine dysregulation in invasive cervical carcinoma and other human neoplasias: time to consider the TH1/TH2 paradigm. *J Natl Cancer Inst* 1998, **90**, 261–263.
36. al-Saleh W, Giannini SL, Jacobs N, *et al*. Correlation of T-helper secretory differentiation and types of antigen-presenting cells in squamous intraepithelial lesions of the uterine cervix. *J Pathol* 1998, **184**, 283–290.
37. van Driel WJ, Kievit-Tyson P, van den Broek LC, Zwinderman AH, Trimbos BJ, Fleuren GJ. Presence of an eosinophilic infiltrate in cervical squamous carcinoma results from a type 2 immune response. *Gynecol Oncol* 1999, **74**, 188–195.
38. Rabinowich H, Suminami Y, Reichert TE, *et al*. Expression of cytokine genes or proteins and signaling molecules in lymphocytes associated with human ovarian carcinoma. *Int J Cancer* 1996, **68**, 276–284.
39. Filgueira L, Zuber M, Merlo A, *et al*. Cytokine gene transcription in renal cell carcinoma. *Br J Surg* 1993, **80**, 1322–1325.
40. Nakagomi H, Pisa P, Pisa EK. Lack of interleukin-2 (IL-2) expression and selective expression of IL-10 mRNA in human renal cell carcinoma. *Int J Cancer* 1995, **63**, 366–371.
41. Piancatelli D, Romano P, Sebastiani P, Adorno D, Casciani CU. Local expression of cytokines in human colorectal carcinoma: evidence of specific interleukin-6 gene expression. *J Immunother* 1999, **22**, 25–32.
42. Schuler T, Kammertoens T, Preiss S, Debs P, Noben-Trauth N, Blankenstein T. Generation of tumor-associated cytotoxic T lymphocytes requires interleukin 4 from CD8(+) T cells. *J Exp Med* 2001, **194**, 1767–1775.
43. Golumbek PT, Azhari R, Jaffee EM, *et al*. Controlled release, biodegradable cytokine depots: a new approach in cancer vaccine design. *Cancer Res* 1993, **53**, 5841–5844.
44. Tepper RI, Coffman RL, Leder P. An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. *Science* 1992, **257**, 548–551.
45. Rodolfo M, Zilocchi C, Accornero P, Cappetti B, Arioli I, Colombo MP. IL-4-transduced tumor cell vaccine induces immunoregulatory type 2 CD8 T lymphocytes that cure lung metastases upon adoptive transfer. *J Immunol* 1999, **163**, 1923–1928.
46. Tartour E, Mehtali M, Sastre-Garau X, *et al*. Phase I clinical trial with IL-2-transfected xenogeneic cells administered in subcutaneous metastatic tumours: clinical and immunological findings. *Br J Cancer* 2000, **83**, 1454–1461.
47. Vowels BR, Lessin SR, Cassin M, *et al*. Th2 cytokine mRNA expression in skin in cutaneous T-cell lymphoma. *J Invest Dermatol* 1994, **103**, 669–673.
48. Gastl GA, Abrams JS, Nanus DM, *et al*. Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. *Int J Cancer* 1993, **55**, 96–101.
49. Wang Q, Redovan C, Tubbs R, *et al*. Selective cytokine gene expression in renal cell carcinoma tumor cells and tumor-infiltrating lymphocytes. *Int J Cancer* 1995, **61**, 780–785.
50. Groux H. An overview of regulatory T cells. *Microbes Infect* 2001, **3**, 883–889.
51. Benchetrit F, Ciree A, Vives V, *et al*. IL-17 inhibits tumor cell growth by means of a T-cell dependent mechanism. *Blood*, 2002, **99**, 2114–2121.
52. Ghosh AK, Moore M. Tumour-infiltrating lymphocytes in cervical carcinoma. *Eur J Cancer* 1992, **28A**, 1910–1916.
53. Tsukui T, Hildesheim A, Schiffman MH, *et al*. Interleukin 2 production in vitro by peripheral lymphocytes in response to human papillomavirus-derived peptides: correlation with cervical pathology. *Cancer Res* 1996, **56**, 3967–3974.
54. Luxton JC, Rowe AJ, Cridland JC, Coletart T, Wilson P, Shepherd PS. Proliferative T cell responses to the human papillomavirus type 16 E7 protein in women with cervical dysplasia and cervical carcinoma and in healthy individuals. *J Gen Virol* 1996, **77**, 1585–1593.
55. Dunbar PR, Smith CL, Chao D. A shift in the phenotype of melan-A-specific CTL identifies melanoma patients with an active tumor-specific immune response. *J Immunol* 2000, **165**, 6644–6652.
56. Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC. Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 1992, **258**, 1795–1798.
57. Bukowski RM, Rayman P, Uzzo R, *et al*. Signal transduction abnormalities in T lymphocytes from patients with advanced renal carcinoma: clinical relevance and effects of cytokine therapy. *Clin Cancer Res* 1998, **4**, 2337–2347.
58. Schmielau J, Nalesnik MA, Finn OJ. Suppressed T-cell receptor zeta chain expression and cytokine production in pancreatic cancer patients. *Clin Cancer Res* 2001, **7**, 933 s-939s.
59. Kono K, Ressing ME, Brandt RM, *et al*. Decreased expression of signal-transducing zeta chain in peripheral T cells and natural killer cells in patients with cervical cancer. *Clin Cancer Res* 1996, **2**, 1825–1828.
60. Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J Immunol* 2000, **165**, 4848–4853.
61. de Visser KE, Kast WM. Effects of TGF-beta on the immune system: implications for cancer immunotherapy. *Leukemia* 1999, **13**, 1188–1199.
62. Sheu BC, Lin RH, Lien HC, Ho HN, Hsu SM, Huang SC. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol* 2001, **167**, 2972–2978.
63. Stanczuk GA, Sibanda EN, Perrey C, *et al*. Cancer of the uterine cervix may be significantly associated with a gene polymorphism coding for increased IL-10 production. *Int J Cancer* 2001, **94**, 792–794.
64. Barth Jr RJ, Camp BJ, Martuscello TA, Dain BJ, Memoli VA. The cytokine microenvironment of human colon carcinoma. Lymphocyte expression of tumor necrosis factor-alpha and interleukin-4 predicts improved survival. *Cancer* 1996, **78**, 1168–1178.
65. Levraud JP, Duffour MT, Cordier L, Perricaudet M, Haddada

H, Kourilsky P. IL-2 gene delivery within an established murine tumor causes its regression without proliferation of preexisting antitumor-specific CTL. *J Immunol* 1997, **158**, 3335–3343.
66. Figlin RA, Thompson JA, Bukowski RM, *et al*. Multicenter, randomized, phase III trial of CD8(+) tumor-infiltrating lymphocytes in combination with recombinant interleukin-2 in metastatic renal cell carcinoma. *J Clin Oncol* 1999, **17**, 2521–2529.
67. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med* 2001, **7**, 1118–1122.