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ARTICLE

The Pattern of Cytokine Gene Expression in Human Colorectal Carcinoma*

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Systemic and local cytokine environment may modulate the immunogenicity of colorectal cancer cells, and affect anti-tumor immune functions of tumor-infiltrating lymphocytes. We therefore investigated cytokine mRNA expression patterns in tumors and peripheral blood mononuclear cells (PBMC) from patients with colorectal adenocarcinoma. IL-2, IFN-γ, tumor necrosis factor-α (TNF-α), IL-4, IL-6, IL-8, IL-10 and IL-1β mRNAs in single cell suspension of freshly isolated colorectal cancer tissue were studied by RT-PCR. Frequencies of cytokine gene expression were compared to those in normal colonic mucosa from tumor patients. The frequencies of IL-2, IFN-γ, IL-4 and IL-10 gene expression were also determined in peripheral blood mononuclear cells from patients with colorectal adenocarcinoma and compared to those of healthy individuals. Tumor samples were more frequently positive for IFN-γ, IL-2, TNF-α and IL-10 gene expression than normal mucosa (p=0.0001, p=0.0118, p=0.001 and p<0.0001, respectively). Frequencies of IL-2 and TNF-α gene expressions were significantly higher in tumors with a diameter <5 cm, than in those with a diameter >5 cm. The genes for IL-6, IL-1β and IL-8 were commonly expressed in both tumor tissue and normal colonic mucosa. IFN-γ transcripts were detected in more PBMC samples from patients with colorectal cancer than those from normal controls (p=0.0449). Thus, colorectal cancer tissue is characterized by a specific pattern of cytokine gene expression. It is likely that multiple interactions between pro- and anti-inflammatory cytokines regulate tumor growth and the functional activity of tumor-infiltrating lymphocytes. (Pathology Oncology Research Vol 10, No 2, 109–116)

Keywords: cytokine, gene expression, colorectal carcinoma, RT-PCR

Introduction

Colorectal adenocarcinoma (CRC) is one of the most frequent and aggressive types of cancer. It is relatively resistant to currently available chemotherapy. Previous attempts to use immune therapy to treat colorectal cancers did not seem to be very effective either,[1] the reason for which is unclear. The anti-tumor immune response is regulated by numerous factors, including cytokines produced by the tumor and other cells in the tumor microenvironment (e.g. epithelial, endothelial cells, tumor-infiltrating leukocytes/TIL). Cytokines can modulate expression and presentation of tumor antigens, adhesion molecules, composition of cellular infiltrate and functional activity of tumor-infiltrating lymphocytes. They can also affect production of immunosuppressive factors by tumor cells. It is plausible that the local cytokine milieu, acting on the tumor cells or on the adjacent cells, can either block or facilitate tumor growth.

Cytokine regulation of human CRC is not clearly understood. Sera of patients with CRC have abnormally high levels of IL-6, IL-4, IL-10, TNF-α and TGF-β1.[2-5] TGF-β and IL-10 have been assumed to play a role in tumor-induced immunosuppression in CRC patients. Impaired cytokine production by mitogen-stimulated peripheral blood mononuclear cells from patients with CRC (IFN-α, IFN-γ, IL-1α, IL-2, IL-12, TNF-α) has also been described.[6-8] Recent studies have demonstrated production or gene

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expression of immunomodulatory cytokines (e.g. TGF-β, IL-10, IL-6, IL-8, IL-7) in various colon carcinoma cell lines or in cancer cells in situ.[9-12] However, there is still no clear explanation for the good prognostic value of cellular infiltrates, especially of intra-tumor NK-cells and $CD8^+$ T-cell infiltrates in human CRC.[13-16] Type 1 helper or cytotoxic T cells (Th1, Tc1, collectively T1 cells) can promote cellular anti-tumor immune functions,[17] whereas cytokines such as IL-10 and IL-4 produced by type 2 helper T or cytotoxic T cells (Th2, Tc2, collectively T2 cells) can inhibit cell-mediated immune responses.[18] Determination of the characteristic cytokine profile (T1, T2) of TIL in CRC seems to be very important. One of the goals of the present study was, therefore, to investigate gene expression of some characteristic T1 (IL-2, IFN-γ) and T2 cytokines (IL-4, IL-10) in tumor tissue from patients with CRC.

As many of the pro-inflammatory cytokines have potent tumor-promoting activity by inducing tumor angiogenesis, synthesis of matrix metalloproteases, or by directly supporting tumor cell growth,[19-24] the present study also aimed to investigate the gene expression of several pro-inflammatory cytokines in tumor tissue. mRNA of a broad spectrum of cytokines (IL-2, IFN-γ, TNF-α, IL-4, IL-6, IL-8, IL-10, IL-1β) was assayed by RT-PCR in freshly isolated tumors from patients with primary CRC. Frequencies of cytokine gene expressions were compared to those in normal colonic mucosa from tumor patients.

Peripheral blood often contains activated lymphocytes during in vivo immune responses.[25,26] Although tumor cells produce immunosuppressive humoral factors that can cause local and systemic immune suppression in advanced tumors,[27] cytokine gene expression in PBMC from tumor patients may be indicative of an ongoing anti-tumor immune response. Peripheral blood mononuclear cells (PBMC) from patients with CRC were, therefore, investigated for type 1 (IL-2, IFN-γ) and type 2 (IL-4, IL-10) cytokine gene expression. PBMC from healthy individuals served as normal controls.

Materials and Methods

Materials

Collagenase (type IV), DNase (type II), and agarose were purchased from Sigma Chemical Co. (St. Louis, MO, USA), anti-CD45-FITC and the IMK kit from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA). TRI reagent was obtained from Molecular Research Center, Inc. (Cincinnati, OH, USA), other molecular biology and cell culture reagents from Boehringer Mannheim (Mannheim, Germany), Pharmacia Biotech, Inc. (Piscataway, NJ, USA), Zenon Biotechnology, Ltd. (Szeged, Hungary), Crosslink Laboratories, Ltd. (Budapest, Hungary), and Gibco-BRL Life Technologies, Inc. (Gathersburg, MD Paisely, Scotland). Oligonucleotides were synthesized by Eurogentec S. A. (Seraing, Belgium) or by Genset Ltd. (France).

Reverse transcriptions and cDNA amplification were performed with a PTC 100 programmable thermal cycler (MJ Research, Inc., Watertown, MA, USA). DNA was visualized, and images were obtained with Gel Doc 1000 transilluminator (Bio-Rad, Hercules, CA). FACS analysis was carried out with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Patients and tumors

Twenty-one patients (13 females, 8 males, mean age: 69.4 years) with primary colorectal adenocarcinoma treated by surgery were included in this study. Patients had not received either chemotherapy or radiotherapy prior to surgery. According to Dukes' staging system, four tumors

Table 1. **Summary of clinical profiles of patients with primary colorectal adenocarcinoma**

	Number of patients	*Mean age (yr)*	*Sex*		*Metastasis*		*Specimen*[2]		
			Female	*Male*	*No*	*Yes*	*T*	*NM*	*PBMC*
Total number of patients	21	69.4	13	8	12	9	17	15	19
Dukes' stage[1]									
A	4	47.5	2	2	4	0	2	1	4
B	8	75.6	6	2	8	0	7	7	7
C	9	73.7	5	4	0	9	8	7	8
Diameter of the tumor									
<5 cm	9	71.3	6	3	8	1	8	7	9
>5 cm	11	67.2	6	5	3	8	8	7	10

[1]Dukes' stage A: infiltrated mucosa, submucosa, or muscular propria
Dukes' stage B: infiltrated intestinal wall with or without infiltrated regional organs
Dukes' stage C: regional or juxtaregional lymph node metastasis
[2]Specimen: T: tumor, NM: normal mucosa, PBMC: peripheral blood mononuclear cell

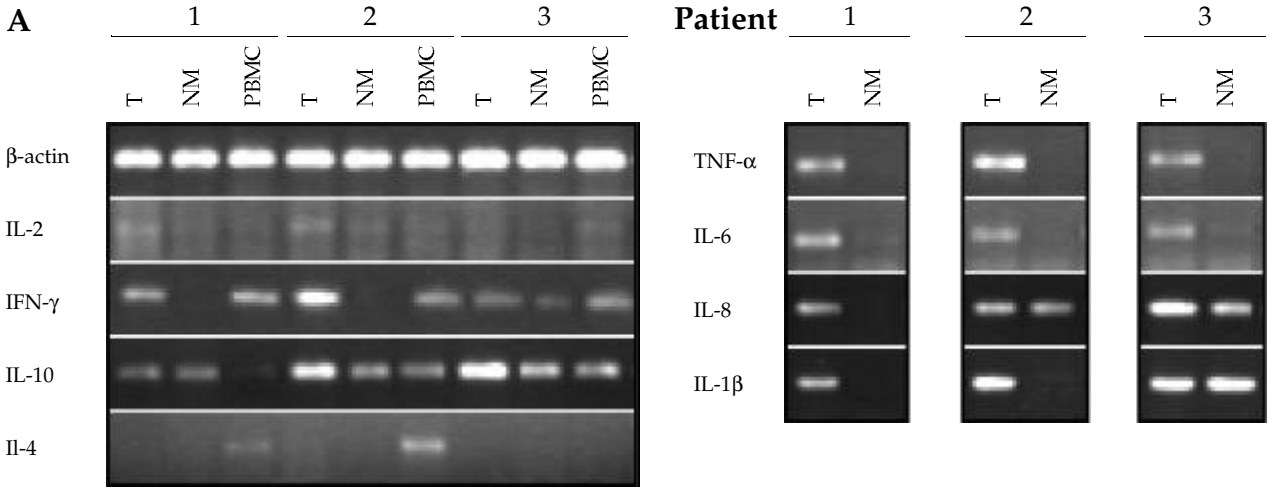

Figure 1. *Representative figure of cytokine gene expression in tumor tissue, normal mucosa and peripheral blood mononuclear cells from patients with colorectal adenocarcinoma and in peripheral blood mononuclear cells from normal controls. Total cellular RNA was isolated from freshly resected primary tumors (T), normal colonic mucosa (NM) and peripheral blood mononuclear cells (PBMC) from three patients with CRC (panel A, Dukes' C: No.1, 2, Dukes' B: No.3) and from PBMC from four healthy controls (panel B) as described in Materials and Methods. The amplified DNAs were resolved in 1.2 % agarose gel containing 0.5 μg/ml ethidium bromide.*

were A (infiltrated mucosa, submucosa, or muscular propria, no metastasis), eight B (infiltrated intestinal wall with or without infiltrated regional organs, no metastasis) and nine C (appearance of regional or juxtaregional lymph node metastases) (*Table 1*). Data on tumor diameter was not available for one case. As normal control tissue, non-affected colonic mucosa specimens were also collected in parallel with the tumor specimens from fourteen patients from the resected tissue, adjacent to the tumor. In 19 cases peripheral blood mononuclear cells (PBMC) from colorectal carcinoma patients were also investigated for cytokine gene expression. PBMC from ten healthy volunteers (1 male and 9 females, mean age 41) were also used as controls.

Tissue samples, cell separation

Single-cell suspensions of the tissue samples were prepared under sterile conditions by enzymatic dissociation as described elsewhere.[28] The chilled cell suspensions were quickly filtered, washed, and assessed for cell recovery (viability>85%). The cells were then resuspended in TRI reagent at 10^7 cells/ml for total cellular RNA preparation.

Blood samples were collected in sterile vacutainer tubes containing 100 U/ml of heparin before pre-medication of the patients, in the morning of the day of the surgical resection. They were placed on ice immediately after sampling. PBMC were prepared by Ficoll-Uromiro cell-density centrifugation as described previously[29]. Cells were washed twice with Hank's solution, counted and resuspended in TRI reagent at 10^7 cells/ml for total cellular RNA preparation.

Cellular RNA isolation

Total cellular RNA was isolated by the method of Chomczynski[30] under RNase-free conditions using TRI reagent (1 ml/10^7 cells), following the instructions of the manufacturer for RT-PCR applications. Contaminating genomic DNA was removed by RNase-free DNase treatment as described elsewhere[29]. For reverse transcription, 3 μg of RNA was reprecipitated with acid/salt/ethanol (1 volume of RNA in aqueous solution 0.08 volume of 3 M Na-acetate, pH 5.4: 3.3 volume of 100% ethanol, –20ºC for 16 h), and dissolved in 1x reverse transcriptase buffer.

Reverse transcription and PCR amplification

Reverse transcription (RT) and PCR were performed with minor modification of the method described by Zou et al.[31] In the first step cDNA was synthesized on a template of 3 μg of cellular RNA, next, cytokine and β-actin cDNA (2 μl of 1/10 diluted stock) were amplified as detailed elsewhere.[28,29] The sequences of sense (S) and antisense (AS) primers, the lengths (L) of the amplicons are as follows.[32] IFN-γ S: GCAGAGCCAAATTGTCTCCT, AS: ATGCTCTTCGACCTCGAAAC, L: 290 bp, IL-2 S: GTCACAAACAGTGCACCTAC, AS: ATGGTTGCTGTCTCATCAGC, L: 262 bp, IL-4: S: ATGGTTGCTGTCTCATCAGC, AS: AACGTACTCTGGTTGGCTTC, L: 224 bp, IL-10 S: ATGCTTCGAGATCTCCGAGA, AS: AAATCGATGACAGCGCCGTA, L: 269 bp, TNF-α S: ACAAGCCTGTAGCCCATGTT, AS: AAAGTAGACCTGCCCAGACT, L: 427 bp, IL-6 S: TCAATGAGGAGACTTGCCTG, AS: GATGAGTTGTCATGTCCTGC,

Table 2. **Cytokine gene expression in tumor and normal colonic mucosa from patients with colorectal carcinoma**

Cytokine	*Tumor (Σn=17[1])*		*Normal mucosa (Σn=15)*		*Significance[2]*
	Positive samples	*%*	*Positive samples*	*%*	*P value*
β-actin	17	100	15	100	ns[3]
IL-2	9	52.9	2	13.3	0.0118
IFN-γ	16	94.1	4	26.7	0.0001
TNF-α	11	64.7	1	6.7	0.001
IL-10	16	94.1	3	20	<0.0001
IL-4	2	11.8	1	6.7	ns
IL-6	12	70.6	5	33.3	ns
IL-8	17	100	14	93.3	ns
IL-1β	10	58.8	6	40.0	ns

[1]total number of samples
[2]significance of difference in cytokine gene expression frequencies between the two groups
[3]ns: not significant

most tumor samples (52.9-100%) (*Table 2*). In contrast, IFN-γ, IL-2, TNF-α, and IL-10 mRNAs were in contrast rare in normal colonic mucosa (p=0.0001, p=0.0118, p=0.001 and p<0.0001, respectively). IL-4 mRNA was similarly rare in both tumor tissue and normal colonic mucosa. Frequencies of IL-2 and TNF-α gene expressions were significantly higher in tumors with Ø<5 cm, than in those with Ø>5 cm (p=0.0406 and p=0.0256, respectively) (*Table 3*). No significant differences were found in the frequencies of cytokine gene expression between groups of patients as a function of Dukes' stage and presence of lymph node metastasis (*Table 3*).

L: 260 bp, IL-8 S: TTGGCAGCCTTCCTGATT, AS: AACTTCTCCACAACCCTCTG, L: 247 bp, IL-1β S: GGATATGGAGCAACAAGTGG, AS: ATGTACCAG-TTGGGGAACTG, L: 263 bp, β-actin S: GGGTCA-GAAGGATTCCTATG, AS: GGTCTCAAACAT-GATCTGGG, L: 237 bp. The primers were specific to cytokine cDNA except those of TNF-α that could be used as an indicator for genomic DNA contamination, resulting in a 727-bp DNA fragment on genomic DNA template.[32] After DNase treatment of RNA specimens, the TNF-α gene specific amplicon could not be observed. The amplified DNA (10 μl) was electrophoresed in a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide in Tris borate/EDTA buffer. Bands were visualized by UV-transillumination. Pictures were analyzed with Molecular Analyst software (Bio-Rad).

Statistical analysis

Fisher's exact test (two-tailed) with a level of significance of p<0.05 was used to compare frequencies of cytokine gene expression between groups of patients and normal controls.

Results

Cytokine mRNA expression in tumor and normal colonic mucosa from patients with CRC

Using RT-PCR, cytokine specific mRNAs were variably, but reproducibly detected in tumor tissue or normal colonic mucosa specimens. A representative ethidium bromide-stained agarose gel with various cytokine specific DNA bands is shown in *Figure 1a*. IL-2, IFN-γ, TNF-α, IL-10, IL-6, IL-1β and IL-8 transcripts were detected in

Cytokine mRNA expression in PBMC from patients with CRC

IFN-γ mRNA was detected in most PBMC samples (84.2%) from CRC patients *(Table 4)*. It was more prevalent in PBMC from patients with CRC than in those from normal controls (p=0.0449) (*Table 4*). IL-2 and IL-10 transcripts were rarely detected in PBMC from patients with CRC and healthy controls (*Table 4*). IL-4 mRNA was observed in a moderate number of samples from both groups (for representative agarose gels see *Figure 1a, b*).

Discussion

Neoplasms produce a complex pattern of soluble factors that are implicated in the immune response against the tumor or in autocrine growth of tumor cells. We investigated local and systemic expression of inflammatory and immunomodulatory cytokines at the tumor site and in the peripheral blood of patients with CRC. Evaluation of cytokine expression at the site of the tumor may give a picture of tumor-host interaction. Evaluation of systemic expression of cytokines helps to elucidate ongoing activation of immune cells in a distance from the tumor.

Expression of IFN-γ and IL-2 is rarely detected in TIL or tumor tissues from numerous types of cancer including CRC.[33-35] We observed specific expression of IFN-γ and IL-2 at the tumor site in human CRC. It is consistent with activated NK and T cells being major effector cell populations in the anti-tumor cell-mediated immune response to human CRC. Their local IFN-γ and IL-2 production may have a direct bearing on the better survival of some patients. In human CRC, large numbers of infiltrating lymphocytes, especially intra-tumor NK cells and $CD8^+$ TIL were reported to be associated with good prognosis of

Table 3. **Cytokine gene expression in tumor tissues from colorectal carcinoma patients with various Dukes' stage, tumor size, and occurrence of lymph node metastasis**

Characteristics of tumors	*Frequency of cytokine gene expression in tumor tissue*															
	IL-2		*IFN-γ*		*TNF-α*		*IL-10*		*IL-4*		*IL-6*		*IL-8*		*IL-1β*	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Dukes' stages																
A (n=2)	1	50	2	100	1	50	2	100	0	0	2	100	2	100	1	50
B (n=7)	5	71.4	7	100	6	85.7	7	100	1	14.3	5	71.4	7	100	4	57.1
C (n=8)	3	37.5	7	87.5	4	50	7	87.5	1	12.5	5	62.5	8	100	5	62.5
Tumor size																
<5 cm (n=8)	7	87.5[1]	8	100	8	100[2]	8	100	2	25	7	87.5	8	100	6	75
>5 cm (n=8)	2	25	7	87.5	3	37.5	7	87.5	0	0	5	62.5	8	100	4	50
Lymph node metastasis																
no (n=9)	6	66.7	9	100	7	77.8	9	100	1	11.1	7	77.8	9	100	5	55.6
yes (n=8)	3	37.5	7	87.5	4	50	7	87.5	1	12.5	5	62.5	8	100	5	62.5

[1]p=0.0406; [2]p=0.0256 (Fischer's exact test)

CRC.[13-16] IFN-γ mRNA abundance in TIL was inversely related to Dukes' stage, and also to levels of TNF-α mRNA in tumor cells.[28]

Our results confirm that TNF-α is an important factor in the tumor microenvironment in human CRC. However, the role of TNF-α in the local regulation of tumor growth and functions of TIL is unclear. In murine adoptive immunotherapy models, secretion of TNF-α and IFN-γ by TIL plays a crucial role in causing tumor regression.[36] Barth et al.[33] reported that better survival of CRC patients was associated with a larger number of TNF-α expressing cells than found in normal mucosa. LPS-induced secretion of TNF-α by cells from human colorectal carcinoma tissue is associated with good prognosis.[37] However, TNF-α may also play a negative role, favoring the growth of colorectal cancer by enhancing neo-vascularization[38] and tumor metastases,[39] and down-regulating the cell-mediated immune response by induction of soluble mediators such as IL-10.[40] A negative correlation between levels of secretion of IFN-γ and TNF-α has been reported in cells cultured from tumor tissue of CRC patients.[35]

Expression of pro-inflammatory cytokines such as IL-6, IL-1β and IL-8 seems to be obligatory, but does not seem to be specific to the tumor microenvironment. However, quantitative differences between mRNA levels of the tumor and normal colonic mucosa cannot be excluded. Specific expression of IL-6 has recently been detected in colorectal tumor tissue.[34] IL-8 mRNA and protein has been reported in situ in colon carcinoma cells and also in infiltrating cells.[9] IL-6 and IL-1β proteins or mRNA, like those of TNF-α are typically undetectable in colon carcinoma cell lines, although there are very few exceptions.[10,41] Our results are supported by data showing that epithelial cells or infiltrating cells in a range of inflammatory bowel disorders,[42] or even in normal colonic mucosa following stimuli like bacterial micro-flora,[43] produce IL-1β, IL-6 and IL-8. Although the role of these cytokines in regulation of tumor growth and spreading in CRC is not fully understood, there is increasing evidence that they support tumor growth or metastasis.[38,39,44-46]

IL-10 mRNA was found in tumor tissue but not in normal colonic mucosa. Secretion of IL-10 may suppress effectors responsible for tumor regression.[47] On the other hand, IL-10 can also suppress the growth and metastatic potential of certain human tumors such as melanoma via inhibition of angiogenesis.[48] IL-10, IL-6, IFN-γ, TNF-α and

Table 4. **Cytokine gene expression in peripheral blood mononuclear cells from patients with colorectal carcinoma and normal healthy volunteers**

Cytokine	*Patients' PBMC* Σ*n=19*[1]		*Control PBMC* Σ*n=10*		*Significance*[2]
	Positive samples	%	*Positive samples*	%	*P value*
β-actin	19	100	10	100	ns[3]
IL-2	4	21.1	1	10	ns
IFN-γ	16	84.2	4	40	0.0449
IL-10	3	15.8	0	0	ns
IL-4	9	47.4	4	40	ns

[1]total number of samples
[2]significance of difference in cytokine gene expression frequencies between PBMC from patients with colorectal carcinoma and normal controls
[3]ns: not significant

IL-1β have been reported to up-regulate IL-10 production in human COLO205 cells.[40] Such interaction between colorectal tumor, adjacent and infiltrating cells may also take place in vivo. In other studies, protein and mRNA of IL-10 was not or was variably detected in tumors from CRC patients.[33,34] The discrepancy between these results is probably due to technical differences and limitations. A significantly larger amount of IL-10 transcripts in freshly isolated tumor cells than in epithelial cells from normal colonic mucosa has been demonstrated previously by our group.[28] IL-4, a Th2/Tc2-type cytokine was not detectable in tumor tissue samples. This argues against locally produced IL-4 regulating tumor progression in human CRC.

We demonstrated that IL-2 and TNF-α gene expression was significantly more frequent in tumors with Ø<5 cm, than in those with Ø>5 cm. Our data are in agreement with previous findings showing that tumors suppress infiltrating macrophage and T cell functions.[49,50] The lack of significant differences in the frequencies of cytokine gene expressions between groups of patients as a function of Dukes' stage could be due to the relatively low number of patients investigated by us.

In our study, IFN-γ mRNA was prevalent in PBMC samples from patients with CRC, suggesting the presence of activated lymphocytes (T and NK cells). Although such in vivo activated cells in the peripheral blood of tumor patients may not be directly involved in anti-tumor immune response, IFN-γ mRNA expression in PBMC from patients might be considered as a potential, useful indicator for cellular activation in relationship to disease progression. Our finding is in accordance with those demonstrating the presence of activated $CD3^+$ T lymphocytes in PBMC from CRC patients[51] and of IFN-γ in some of the patients' sera.[2] It raises the possibility that detection of IFN-γ mRNAs in peripheral blood can be a useful tool for monitoring immune activation status of patients with CRC.

Only few of our PBMC samples from tumor patients contained detectable IL-10 mRNA, suggesting that the source of systemic IL-10 demonstrated by several authors in association with progression of the disease[3,52] is not PBMC. IL-4 mRNA was found in some PBMC samples from both patients with CRC and normal controls, consistent with our previous fndings.[29] It can be associated to the large number of women among these individuals. Th1/Th2 cytokine production is affected by sex hormones.[53] High IL-4 protein levels in sera from patients with CRC were demonstrated in two studies.[2,5] However, our study does not exclude the possibility that the level of IL-4 mRNA expression is higher in PBMC from CRC patients than in those from normal controls.

It is unlikely that the cytokine gene expression observed in tumor tissue is caused by peripheral blood contamination, because mRNAs of certain cytokines, as IL-2 were more prevalent in tumor tissue than in PBMC from the patients.

In conclusion, we describe the pattern of cytokine gene expression associated with human CRC. The local tumor microenvironment is characterized by simultaneous expression of various cytokines that may form a complicated regulatory network. Local expression of immunomodulatory cytokines like IFN-γ and IL-2 at the tumor site suggests involvement of specific T (Th1/Tc1) or NK cells in the local anti-tumor immune response. An ongoing anti-tumor immune response in a number of patients with CRC could be supported by the presence of activated mononuclear cells expressing IFN-γ mRNAs in the peripheral blood. However, the local or systemic presence of pro-inflammatory cytokines and their potential interactions with other cytokines may favor tumor progression.[40,54,55,] Our study indicates that the gene expression of type 1 cytokines, particularly that of IL-2 in the tumor micromilieu seems to be down-regulated as a function of the tumor mass. Quantitative determinations of cytokine mRNAs (or proteins) would undoubtedly help elucidate the net effects of interactions between cytokines and predict the progression of disease.

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