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Characterisation of cytokine mRNA expression in tumour-infiltrating mononuclear cells and tumour cells freshly isolated from human colorectal carcinomas.

Abstract

The in situ function of tumour-infiltrating leukocytes (TIL) in human colorectal adenocarcinoma (CRC) is unclear. Local cytokine expression probably regulates the anti-tumour immune response and tumour immune surveillance. We examined the distribution of mRNA for IFN-g, TNF-a, IL-10 and IL-4 in TIL, and

tumour cells freshly isolated from 21 surgically removed primary CRC, using a semiquantitative RT-PCR. Lamina propria-infiltrating leukocytes (LPL) and epithelial cells from normal colon mucosa of 10 CRC patients served as negative controls. Median levels of IFN-g and TNF-a mRNA were higher in TIL than LPL (p = 0.0002 and 0.0001). IL-10 mRNA was generally observed in TIL and LPL, but no or very small amounts of IL-4 transcripts were detected in TIL and LPL. TNF-a and IL-10 mRNA were more abundant in colorectal tumour cells than in the normal epithelial cells (p = 0.0136 and 0.0036). The number of IFN-g transcripts in TIL correlated negatively (p = 0.039) and the number of TNF-a transcripts in tumour cells correlated positively with the Dukes' stages (p = 0.0147). Our results suggest that TIL are characterized by a type 1 (Th1/Tc1-like) pattern of cytokine expression and function as T cells (and macrophages) in the local, cell-mediated anti-tumour immune response in early stages of CRC. Changes in IFN-g and TNF-a mRNA in TIL and tumour cells could be related to tumour progress (e.g. by T cell anergy) or forming of metastases, respectively.

Full text

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent and aggressive neoplasms, for which the adjuvant chemotherapy after surgery has little success. Immunotherapy (rIL-2 alone or in combination with lymphokine-activated killer cells, IFN-alpha and IFN-gamma combined with 5-fluorouracil and folinic acid) also has had only a small or no effect in patients with advanced CRC [1], despite adoptive transfer of TIL having been shown to induce tumour regression and inhibit metastasis in general, in experimental animal models [2, 3].

The majority of human CRC are extensively infiltrated by lymphoid and monocytic cells and the presence of such infiltrates is associated with improved survival [4-7]. Intratumoral NK and $CD8^+$ T cell infiltrate have also recently been shown to be prognostic markers in CRC [8, 9]. In contrast, isolated colon carcinoma-infiltrating lymphocytes (TIL) being able to proliferate in response to IL-2 *in vitro*, fail to kill autologous tumour cells [10-12]. This suggests that their beneficial effect is mediated by a mechanism other than direct cytotoxicity against tumour cells.

Cytokines are major regulators of the local immune responses that can lead both to regression and progression of tumour growth by acting on the tumour cells directly, or the adjacent cells. They can activate T cell subsets, NK cells, macrophages, dendritic cells, and modify immunogenicity and growth of malignant cells. Secretion of cytokines (*e.g.* IFN-gamma, IL-2, TNF-alpha) by type 1 helper T (Th1) cells in response to tumour cell antigens can promote cellular anti-tumour immune functions [13]. Cytokines such as IL-10 and IL-4 produced by type 2 helper T (Th2) cells can suppress cytokine production by antigen-presenting cells and Th1 cells, resulting in inhibition of cell-mediated immune responses, enhancing tumour growth. $CD8^+$ T cells, a major effector cell population mediating resistance to cancer, have also been classified into two distinct effector cell types based on their cytokine-secreting profiles [14]. The patterns of cytokine secretion by type 1 (Tc1 cells) and type 2 (Tc2) $CD8^+$ T cells are similar to those of Th1 and Th2 cells, respectively. Their role in tumour immunity is less well characterized. Recently, promotion of long-term tumour immunity by Tc1 and Tc2 cells *via* different mechanisms has been described in a murine lung metastases/adoptive transfer model [15].

The role of tumour-infiltrating mononuclear cells in the anti-tumour host response is a matter of debate for several reasons [13, 16]. Although tumours are often infiltrated by $CD4^+$ and $CD8^+$ T lymphocytes and macrophages, that can produce the cytokines necessary for effective, cell-mediated anti-tumour responses, tumour-infitrating cells can also secret soluble mediators (*e.g.* heparin-binding epidermal growth factor, platelet-derived endothelial cell growth factor, basic fibroblast growth factor, vascular endothelial growth factor) that promote tumour progress directly or indirectly, by inducing vascularisation and metastasis for example [13, 17-20]. TIL freshly isolated from human tumour tissues are frequently defective in their anti-tumour responses and signal transduction *via* the TCR-CD3 receptor complex [16, 21], and it has been demonstrated that tumour-associated macrophages are in a deactivated state [22]. Elimination or depletion of tumour-antigen-reactive T cells from the tumour microenvironment, anergy of tumour specific T cells, and unbalanced production of cytokines at the site of the tumour, all influencing T cell regulatory functions, are believed to be responsible for defective effector functions of TIL. However, which cytokines are produced by TIL *in situ*, and how they cooperate in the course of the disease needs to be determined for each type of tumour.

Cytokine regulation of human CRC is not well understood. Recent studies have demonstrated cytokine production by various colon carcinoma cell lines and cancer cells (*e.g.* TGF-beta, IL-10, IL-6, IL-8, IL-7) [23-26] indicating that tumour cell-derived factors can influence cell-mediated and humoral immune responses against tumour cells. TGF-beta and IL-10 are probably involved in tumour-induced immunosuppression in CRC patients. Cytokine production by TIL isolated from human CRC has not been clearly described with respect to Th1 and Th2 cytokine profiles. Serum cytokine levels in patients with CRC can be informative for systemic [27, 28], but not for local effects of cytokines. Also, due to the lower sensitivity of blood tests, the results do not always indicate the progression of the disease. Cytokine expression *in situ* - the most relevant to local immune regulation of tumor growth and metastasis - has drawn less attention as regards establishing an association between prognosis and local immune status in human CRC. Characterization of the potential of TIL to support cell-mediated anti-tumour host responses, and the information about the local cytokine microenvironment in CRC would therefore be valuable. The aim of this study was to investigate cytokine mRNA, particularly those for IFN-gamma, TNF-alpha (type 1 or Th1/Tc1-type cytokines), IL-10 and IL-4 (type 2 or Th2/Tc2-type cytokines), in tumour-infiltrating leukocytes and tumour cells freshly isolated from patients with CRC. A sensitive, semiquantitative technique was used: RT-PCR.

MATERIALS AND METHODS

Materials

Collagenase (type IV), and DNase (type II) were purchased from Sigma Chemical Co. (St. Louis, MA, USA), $CD45^+$ microbeads, and separation columns (MiniMACS starting kit) from Miltenyi Biotec GmbH. (Bergisch Gladbach, Germany), and 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), RNase-free DNase, human placental RNase inhibitor (HPRI), oligo-dT_{12-18}, streptavidin-coated 96-well microtitre plates, digoxigenin-dUTP, peroxidase-labelled anti-digoxigenin monoclonal antibody, and ABTS from Boehringer Mannheim (Mannheim, Germany), dNTP from Pharmacia Biotech, Inc. (Piscataway, NJ, USA), Taq polymerase (Dupl-A-Taq) from Zenon Biotechnology, Ltd. (Szeged, Hungary), 10 x Taq buffer (CrossTaq buffer) Crosslink Laboratories, Ltd. (Budapest, Hungary), and

Moloney murine leukemia virus reverse transcriptase (M-MLV RT), RPMI-1640, and glutamine from Gibco-BRL Life Technologies, Inc. (Gathersburg, MD; Paisley, Scotland). Oligonucleotides were synthesised by Eurogentec S. A. (Seraing, Belgium) or Genset, Ltd. (France). pQA1 and pQB3 are plasmids containing multispecific synthetic competitor sequences and were kindly provided by Dr. D. Shire (Sanofi Elf Bio Recherches., Labège, France).

FACS analysis was carried out with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA), nucleic acid concentrations were determined with a GeneQuantII UV-spectrophotometer (Pharmacia Biotech, Cambridge, England), reverse transcriptions, DNA amplifications and synthesis of biotin-labelled single strand DNAs were performed with a PTC 100 programmable thermal cycler (MJ Research, Inc., Watertown, MA, USA). Optical density at 405 nm was measured with an Anthos HT II microplate reader (Anthos Labtec Instruments, Salzburg, Austria).

Patients and tumours

In total, twenty one patients (11 females, 10 males, mean age: 68.5 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' classification, five patients were classifed as stage A (infiltrated mucosa, submucosa, or muscular propria, no metastasis), seven as stage B (infiltrated intestinal wall with or without infiltrated regional organs, no metastasis) and 8 as stage C (appearance of regional or juxtaregional lymph node metastases) (Table 1). The result of the histological investigation was not available for one case such that the Dukes' classification of the tumour was not possible. As normal control tissue, nonaffected colon mucosa specimens were also collected from the resected area in ten patients, adjacent to the tumour, in parallel with the tumour specimens.

Isolation of TIL, normal lamina propria-infiltrating lymphocytes (LPL), tumour cells and normal epithelial cells (EC)

Single-cell suspensions from the tumour tissue and normal intestinal mucosa were prepared under sterile conditions with minor modification of the method described by Itoh *et al.* [29]. Briefly, the freshly dissected tumour samples were cleaned of necrotic areas, fat and normal tissues. These samples and the normal

colon mucosa specimens, were washed with tissue culture medium (RPMI-1640), then minced into 1-3 mm^3 pieces. Single cell suspensions were obtained by enzymatic dissociation of the tissues by digesting the cells with collagenase type IV and DNase (10 ml/g tissue of 100 U/ml of collagenase type IV, 77 U/ml of DNase type II in serum-free RPMI-1640 supplemented with 40 mug/ml gentamicin) at 37° C for 1.5 hour. The cell suspensions were then gauze-filtered, washed, and resuspended in RPMI-1640 for assessment of cell recovery and viability (>85%). Tumour-infiltrating and lamina propria-infiltrating lymphocytes were further enriched by Ficoll-Uromiro cell-density centrifugation. The cells were then counted and processed for magnetic cell sorting for $CD45^+$ cells, according to the manufacturer's instructions (Miltenyi Biotec, MiniMACS kit).

In short, viable (> 95%), pre-filtered cells, up to 10^7, were labelled with 1:5-diluted anti-CD45-microbeads in 100 mul PBS containing 0.5% BSA/2 mM EDTA/0.01% Na-azide at 4° C for 20 min, then washed and passed over a separation column (type MS) under a magnetic field. The positively selected cell populations, of which > 85% were $CD45^+$ cells, were considered to be TIL and LPL. The $CD4^+$-depleted cells in the flow-through (> 95% $CD45^-$ cells) were considered to be tumour or normal epithelial cells, respectively. Cell surface analysis of TIL by immunostaining with the IMK panel in a few, unselected cases showed that the population gated for lymphocytes in FSC/SSC parameters (91.4 ± 7.5% $CD45^+$ cells in total) was 16.7 ± 16.1% $CD45^+/CD14^+$, 63.6 ± 18.6% $CD3^+/CD19^-$, 24.5 ± 23.9 $CD4^+/CD8^-$, 21.0 ± 21.2 $CD4^-/CD8^+$, 15.1 ± 11.7% $CD3^-/CD19^+$, 6.0 ± 6.9% $CD3^-/CD16^+/56^+$, 21.6 ± 20.4% $CD3^+/CD16^+/56^+$, 51.0 ± 30.0% $CD3^+$/HLA-DR^+ (n = 6, mean ± SD). FACS analysis was carried out using Ficoll-Uromiro enriched TIL before magnetic cell sorting because of the interference between the fluorescence derived from magnetic beads and phycoerythrin-conjugated antibodies.

Cellular RNA preparation

Total cellular RNA was isolated by the method of Chomczynski [30] under RNAse-free conditions using TRI reagent (1 ml/1 x 10^7 cells) following the manufacturer's instructions for RT-PCR applications. Contaminating genomic DNA was removed by RNase-free DNase treatment (10 units of DNAse/RNA from 5 x 10^6 cells, at 37° C for 10 min) in the presence of 2.5 units of human placental RNase inhibitor, followed by repurifying the RNA by the protocol used for RNA isolation with TRI reagent. For reverse transcription, 3 mug of RNA was

reprecipitated with acid/salt/ethanol (1 vol of RNA in aqueous solution : 0.08 volume 3M Na-acetate, pH 5.4: 3.3 volume 100% ethanol) by incubation at - 20° C for 16 hours, and then dissolved in 1x reverse transcriptase buffer.

Reverse transcription and competitive PCR

Reverse transcription (RT) and competitive PCR was performed with minor modification of the method described by Zou *et al.* [31]. In the first step, cDNA was synthesized in a reaction mixture (20 mul) containing 3 mug of celllular RNA, 0.5 mug (16 pmoles) of oligo(dT)$_{12-18}$, 10mM dithiotreitol, 0.5 mM dNTP, 200 units of Moloney murine leukemia virus reverse transcriptase and 40 units of human placental RNase at 42° C for 60 min, followed by denaturing cDNA at 94° C for 5 min. cDNA was kept at - 80° C until DNA amplification. DNA amplifications were carried out in the presence of linearized pQA1 and pQB3 plasmids containing multispecific competitor sequences of synthetic origin. pQA1, pQB3 and other members of the pQA and pQB family of plasmids have previously been used for analyzing cytokine gene expression by quantitative and semiquantitative RT-PCR [31, 32]. We used pQA1 to compete with IFN-gamma, IL-4, and TNF-specific DNA amplification, and pQB3 to compete with that of IL-10 and beta-actin DNA. The sequences of the sense and antisense primers used, and the lengths of the cDNA- and competitor-specific amplicons are listed in Table 2.

beta-actin cDNA (2 mul) was first quantified by competitive PCR with a series of concentrations (0.01, 0.1, 1, 10 pmoles) of linearized pQB3. The reaction mixture (30 mul) consisted of 300 nM of sense and antisense primers, 0.045U/mul Taq polymerase, 50 muM of each dNTP in 1x Taq buffer containing 2.5 mM $MgCl_2$. Amplification was performed with the following program: 1 cycle of 94° C for 5 min, 57° C for 1 min, 72° C 1.5 min; 31 cycles of 94° C for 1 min, 57° C for 1 min, 72° C for 1.5 min; 1 cycle of 94° C for 5 min, 57° C for 1 min, 72° C 5 min. Next, cytokine cDNA were coamplified with cDNA preparations containing 10^6 molecules of beta-actin cDNA and one of a series of concentrations of competitor plasmid pQB3 (0.01, 0.1, 1, 10 pmoles) or pQA1 (0.001, 0.01, 0.1, 1 pmoles) under the same conditions as for beta-actin, except that instead of 31 cycles, 35 cycles of PCR were performed, and the synthesis was at 55° C.

The amplified cDNA-specific and competitor DNA-specific products were quantified by a colorimetric assay [31]. In short, in the first step a digoxigenin-dUTP-labeled DNA strand was synthesized from the competitive PCR products

(1.4 mul) in the presence of 5 muM dNTP, 250 nM digoxigenin-dUTP, 0.2 U Taq polymerase, 300 nM 5'-biotinylated oligonucleotide recognizing either the amplified cDNA or the amplified competitor, and 1x Taq buffer in 20 mul final volume using a single PCR cycle of 94° C for 5 min, 60° C for 1 min, and 72° C for 2 min, 94° C 2 min. The sequences of the 5'-biotinylated oligonucleotides are shown in the Table 2. The labeled DNA (1.5 mul) was quantified using an ELISA-type assay after binding the DNA to streptavidin-coated microtiter plates in 200 mul 0.2% Tween/PBS supplemented with 1% low-fat milk powder (3 hours, 37° C), followed by incubation with 200 mul of 1/15000-diluted peroxidase-conjugated anti-digoxigenin monoclonal antibody at 37° C for 30 min. The reaction was developed with ABTS substrate. The optical density was measured at 405 nm. Under these conditions, a strict linearity was observed between the log ratio of amplified cDNA/competitor DNA molecules and the log number of competitor DNAs in each case ($r^2 > 0.9$). Numbers of amplified cDNA molecules were calculated from the equivalence point of the linear regression curve.

Statistical analysis

The numbers of DNA molecules (referred to hereafter as "transcripts") amplified from TIL and LPL, tumour cells and normal mucosa epithelial cells, TIL and tumour cells, as well as LPL and epithelial cells were compared using the non-parametric Mann Whitney test, with a level of significance of $P < 0.05$. Correlations between Dukes' staging and numbers of DNA molecules were determined by Spearman rank order correlation ($P < 0.05$).

RESULTS

Expression of the IFN-gamma *gene in TIL and tumour cells from colorectal adenocarcinoma*

IFN-gamma mRNA was detected by competitive PCR using the pQA1 competitor in 100% of TIL and 80% of LPL samples. The number of transcripts in the TIL was significantly higher than that in the LPL. (Figure 1). Although IFN-gamma gene expression was detected in 39% and 22% of tumour cell and normal mucosa epithelial cell samples, the level of expression was in most cases very low. The number of transcripts in the TIL was significantly higher than that in the tumour cell population. No significant difference was observed in IFN-gamma gene

expression between LPL and fractions of normal mucosa epithelial cells, or between fractions of tumour cells and normal mucosa epithelial cells.

TIL from patients with tumours at Dukes' stages A, B or C contained IFN-gamma mRNA with the amount decreasing from stage A to C (Figure 2). IFN-gamma production in TIL showed a negative correlation with stages A, B and C ($r = -0.501$, $p = 0.0390$), and the difference between the stages A/B and C was significant.

*TNF-*alpha *gene expression in TIL and tumour cells from colorectal adenocarcinoma*

TNF-gamma mRNA was quantified by competitive PCR using the pQA1 competitor

We detected TNF-alpha mRNA expression in 53%, 20%, 78%, and 22% of samples of TIL, LPL, tumour cells and normal mucosa epithelial cells, respectively. The number of TNF-alpha transcripts was significantly higher in TIL than in LPL (Figure 3). The tumour cells contained significantly more TNF-alpha mRNA than did normal mucosa epithelial cells. The number of mRNA transcripts in TIL and tumour cells did not differ significantly. Tumour cells from patients with tumours at Dukes' stages A, B and C contained TNF-alpha mRNA in amounts increasing from stage A to C (Figure 4). We found a significant correlation between the number of TNF-alpha transcripts in tumour cells and the Dukes' stage ($r = 0.580$, $p = 0.0147$), and the difference in TNF-alpha expression between stages A/B and C was also significant (Figure 4).

IL-10 mRNA in TIL and tumour cells from colorectal adenocarcinoma

IL-10 mRNA was quantified by competitive PCR using the pQB3 competitor. We detected IL-10 mRNA in 84% of samples of TIL, 60% of LPL, 78% of tumour cells and in 56% of normal mucosa epithelial cells. The numbers of IL-10 transcripts in TIL, LPL and the tumour cell population were not significantly different (Figure 5). There was however more IL-10 mRNA in the tumour cell population than in the normal epithelial cells.

There were no significant differences in IL-10 gene expression between the stages nor was any correlation between the amount of mRNA in TIL or tumour

cells and the Dukes' stages observed.

IL-4 mRNA in TIL and tumour cells from colorectal adenocarcinoma

We determined IL-4 mRNA levels by competitive PCR using the pQA1 competitor.

IL-4 mRNA levels were very low, or undetectable, in LPL and mucosa epithelial cells. We found small numbers of IL-4 transcripts in 24% of TIL and 19% of tumour cell samples. The number of transcripts did not differ significantly between any of the cell populations investigated. There was no significant correlation between the IL-4 mRNA level in TIL or tumour cells, and the stages of the disease (Figure 6).

DISCUSSION

Our results suggest that tumour-infiltrating cells in human CRC have a particular pattern of cytokine expression at the site of the tumour. We demonstrate that TIL freshly isolated from CRC express genes for IFN-gamma and TNF-alpha at higher levels than those found for infiltrating mononuclear cells (LPL) in the corresponding normal compartment, *i.e.* normal colon mucosa. In addition, expression of IFN-gamma in TIL, and expression of TNF-alpha in tumour cells correlated with the Dukes' stage of the disease negatively and positively, respectively.

The major sources of IFN-gamma are activated $CD4^+$ Th1-type and $CD8^+$ Tc1-type lymphocytes, and NK cells. IFN-gamma influences all major functions of macrophages, including cytokine production, tumour cell cytotoxicity, phagocytosis and antigen-presentation *e.g.* by up-regulation of HLA-DR antigen on antigen presenting cells. It also enhances NK cell-mediated cytotoxicity, and immunogenicity of colon carcinoma cells [33, 34]. Human colon carcinoma cells are sensitive to *in vitro* apoptosis induced by IFN-gamma and TNF-alpha [35]. IFN-gamma expression can also directly contribute to colon tumour regression by inhibition of tumour cell invasion and metastasis (*e.g. via* suppression of the expression of S100A4 calcium-binding protein) in mouse [36]. In human CRC, large numbers of infiltrating lymphocytes has been reported to be associated with a good prognosis of CRC [4-7], and intratumoral NK cells and $CD8^+$ TIL were found to be associated with a better prognosis [8, 9]. Therefore our findings are consistent with activated NK cells and T cells (Th1/Tc1) being major effector cell

populations in the anti-tumour, cell-mediated immune response to human CRC, and their local IFN-gamma production may be related directly to improved survival.

TNF-alpha is produced by many cell types, including activated macrophages, monocytes, T cells (mostly Th1/Tc1 cells), *e.g.* T cell clones obtained from colon carcinomas [10], and various tumour cells [37].
TNF-alpha mediates many potent anti-tumour effects, including the induction of tumour regression *in vivo* alone or with adoptively transferred TIL, or genetically modified tumour cells in animal models. It activates macrophages and supports TIL proliferation [38, 39]. Synergistic anti-tumour effects of TNF-alpha and IFN-gamma have also been demonstrated *in vitro* on human colorectal adenocarcinoma cells, and *in vivo* in mice [35, 40, 41]. Our results do not exclude the possibility that TNF-alpha produced by TIL also participates in a beneficial, anti-tumour host response, at least in less advanced stages of CRC. As the distribution of TNF-alpha expression in $CD45^+$ TIL in different Dukes'stages does not coincide with that of IFN-gamma, at least one major source of TNF-alpha in TIL is not IFN-gamma secreting cells. TNF-alpha may also be derived from a small percentage of macrophages ($CD14^+$ cells) within the $CD45^+$ TIL population.

IFN-gamma expression in TIL or tumour tissues is variable, and rare in freshly excised human metastatic melanoma and renal cell carcinoma as assessed by RT-PCR [33, 42, 43]. TNF-alpha mRNA has been found in freshly isolated TIL, tumour cells, and normal renal tissue in human renal cell carcinoma [44]. In a human colon tumour/tissue-isolated rat model, adoptively transferred human activated NK cells infiltrating the tumour showed immunostaining *in situ* for TNF-alpha in one-third of the specimens [45]. Barth *et al.* [6], detected a small percentage of IFN-gamma secreting cells, and a higher percentage of TNF-alpha-producing $CD3^+$ cells in TIL from human primary CRC on paraffin-embedded sections by immunohistochemistry. They found an association between patient' survival and the number of TNF-alpha-secreting, but not the number of IFN-gamma-secreting TIL. These data seem discordant with ours that show a negative correlation between IFN-gamma gene expression in TIL and Dukes' stage, and no correlation of TNF-alpha expression with stage. The discrepancy could be due to the different methods used and their different sensitivities. Also, although in general, survival correlates with Dukes' stages, the two variables are not the same. Insufficient time has passed since our study was conducted to allow valid analysis of the association between the levels of

cytokine expression and survival. However, our results are in concordance with the findings of Lahm *et al.* [46] who observed that IFN-gamma and IL-2 production in mitogen-activated whole blood cell cultures correlated negatively with the progression of CRC.

Our data suggest that IFN-gamma expression by TIL supports an anti-tumour host response mostly in the early stages of CRC. The decrease in the IFN-gamma gene expression in TIL from patients with advanced stages of CRC may be caused by T cell anergy [47] or selective loss of type-1 T cells from TIL during tumor growth [48]. Alteration of antigen-specific signal transducing molecules (CD3dzeta) in TIL [21] may also be responsible for decreased IFN-gamma mRNA expression by impairment of the IFN-gamma induction pathway.

We also found TNF-alpha transcripts (but not IFN-gamma mRNA) in tumour cells. There was a positive correlation between the level of TNF-alpha expression in tumour cells and Dukes' stage, and this suggests that TNF-alpha may mediate tumour progress in human CRC. It is unlikely that the TNF-alpha transcripts in the tumour cell fractions were from a small percentage of contaminating $CD45^+$ cells (although this possibility cannot be excluded) because IFN-gamma transcripts (representing $CD45^+$ contamination) were found in only a small number of tumour samples. The reason why TNF-alpha expression in tumour cells increases during the course of the disease is unclear: it may be due to positive selection of a TNF-alpha resistant tumour cell population, or enhanced stimulation of TNF-alpha expression by the tumour cells under the influence of changing tumour micro-environment.

Several studies have demonstrated the role of TNF-alpha in tumour progress. TNF-alpha induces the expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin, $beta_2$-integrin) on endothelial cells, which in turn promote the adhesion of colorectal tumour cells to the endothelium enhancing the metastatic potential of the tumour [49]. TNF-alpha can also promote angiogenesis in solid tumours such as human malignant melanoma, prostate cancer through induction of IL-8 and VEGF secretion by melanoma cells [20, 50, 51].

IL-10 produced by tumour cells or TIL is involved in the escape by malignant cells from immune surveillance. IL-10 can downregulate antigen-presenting, cytokine expression (*e.g.* IL-12), and anti-tumour activities of monocytes by inhibiting the

production of anti-tumour effector molecules, it blocks cell-mediated effector cell functions by inhibiting cytokine secretion (*e.g.* IFN-gamma, TNF-alpha) in Th1 cells, and can also protect tumour cells from CTL mediated lysis [52]. Its elevated expression in various human tumours [52], and its elevated serum level in human CRC [27] indicate its important regulatory role in the regulation of the anti-tumour immune response. However, IL-10 can also suppress the growth and metastatic potential of certain human tumours such as melanoma and breast carcinoma *via* inhibition of angiogenesis [53]. Our results show that IL-10 expression is characteristic of TIL and LPL, with a (non-significantly) higher average IL-10 expression in TIL than LPL. IL-10 mRNA expression in none of the cell populations correlated with Dukes' stages. IL-10 is produced primarily by activated Th2/Tc2 lymphocytes, activated monocytes, macrophages, and also many other cell types including activated B cells, epithelial cells, and tumour cells. In our study, the major sources of IL-10 in $CD45^+$ TIL and LPL may have been T cells (Th2/Tc2) and macrophages. We detected IL-10 transcripts not only in TIL and normal colon LPL, but also in the tumour cells. This is in agreement with other data for CRC or normal colon epithelium [6, 25, 54, 55], although the presence of small amounts of $CD45^+$ cell contamination in the tumour cell fractions cannot be fully excluded. The expression of IFN-gamma in TIL in less advanced stages of CRC, and the lack of correlation between IL-10 expression in TIL and Dukes' stage observed in our study would argue against any substantial effect of IL-10 on local T cell anergy in CRC.

IL-4 is produced primarily by activated Th2/Tc2 cells, and is another candidate regulator of anti-tumour immune responses. IL-4 was shown to have direct anti-proliferative effects on human colon carcinoma cells *in vitro* [56]. Its anti-tumour effects have been demonstrated *in vivo* by retroviral gene transfer into colon carcinoma cells [57] in mice, and in an $IL\text{-}4^{(-/-)}$ mouse model [58]. In contrast to the IL-10 gene expression, we observed very low level or undetectable levels of IL-4 mRNA in TIL, tumour cells, normal colon mucosa LPL and epithelial cells. This is inconsistent with IL-4 contributing to the local regulation of the cell-mediated anti-tumour response, or to tumour progress by acting directly on tumour cells in human CRC. Piancatelli *et al.* [55] also reported no detectable IL-4 mRNA in freshly isolated tumour tissues and normal colon mucosa from CRC patients. Barth *et al.* [6] found that a higher frequency of IL-4 secreting cells (in addition to an increase in the number of TNF-alpha producing cells) in TIL, detected by immunohistochemistry, was associated with improved survival in primary CRC. The apparent discrepancy between these results and those of IL-4 gene

expression is probably due to technical limitations. We could detect large amounts of IL-4 transcript in PHA-stimulated peripheral blood lymphocyes (data not shown) indicating that the method used by us was sensitive for detection of IL-4 mRNA.

CONCLUSION

In general, our results suggest that CRC-infiltrating mononuclear cells at the site of the tumour express cytokines characteristic of inflammation (IFN-gamma, TNF-alpha). Increased expression of IFN-gamma by TIL provides a survival advantage to the increased number of $CD8^+$ cells and NK cells in the infiltrate observed previously [8, 9]. We suggest that anergy or depletion of Th1/Tc1 cells, and increasing amounts of TNF-alpha at the site of the tumour in more advanced stages of CRC, in the continuous presence of certain level, of IL-10 (and/or of various other soluble factors) provide a microenvironment that supports tumour progress.

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Figures

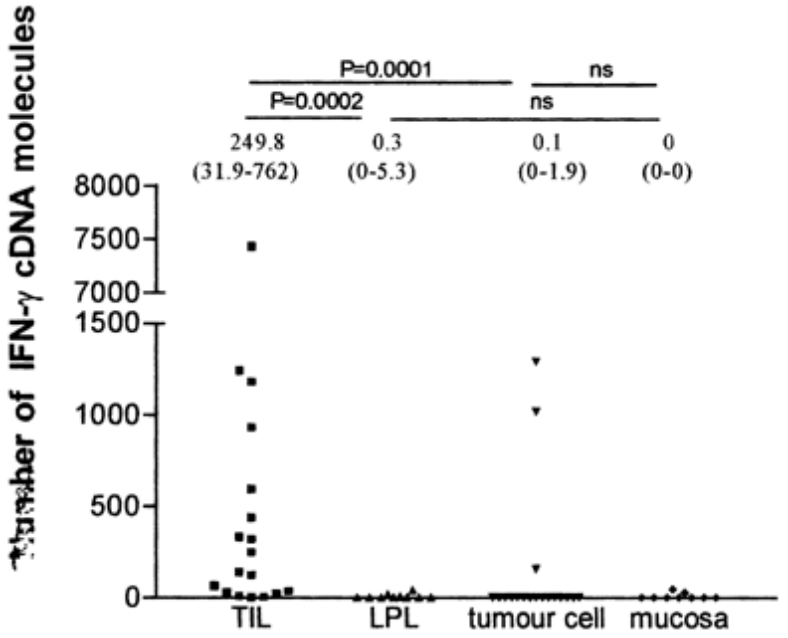


Figure 1

IFN-gamma mRNA in cells from tumour and normal colon mucosa.

IFN-gamma transcripts were quantified by a semiquantitative RT-PCR using pQA1 competitor DNA in TIL (n = 19), tumour cells (n = 18), lamina propria-infiltrating leukocytes (LPL) (n = 10) and epithelial cells in normal mucosa (n = 9) from patients with colorectal carcinoma as described in Materials and Methods. Values are numbers of IFN-gamma cDNA/10^6 beta-actin cDNA molecules. Median numbers of IFN-gamma-specific DNA amplicons/10^6 beta-actin DNA amplicons with 25 and 75% percentiles in parenthesis are indicated above the scatters of each cell population. Significance differences between cell populations was calculated with the Mann Whitney test.

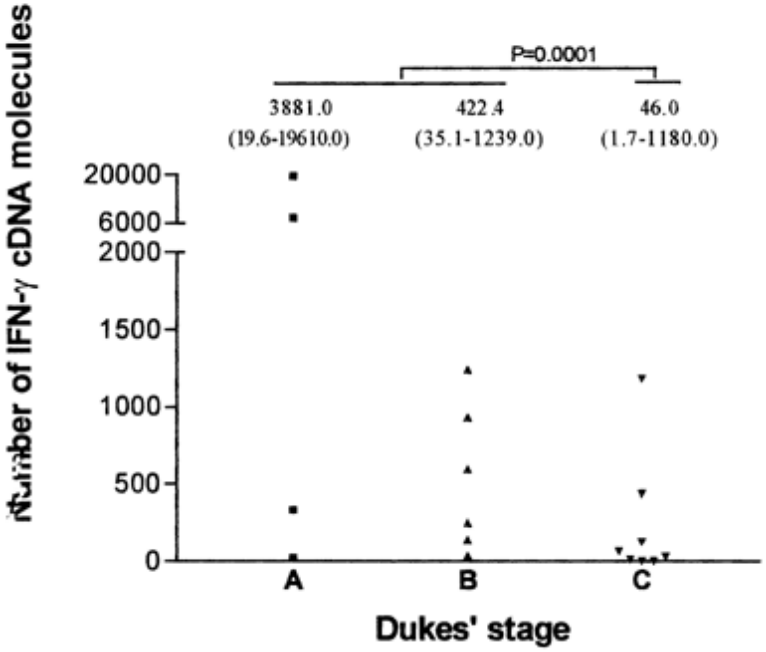


Figure 2

IFN-gamma mRNA in TIL from different stages of colorectal cancer.

Amplified IFN-gamma cDNA was quantified by competitive PCR in TIL from patients with Dukes' A (n = 4), B (n = 6) and C (n = 8) stages of colorectal carcinoma as described in Materials and Methods. Values are numbers of IFN-gamma cDNA/10^6 beta-actin cDNA molecules. Median numbers of IFN-gamma specific DNA amplicons/10^6 beta-actin DNA amplicons with the minimum and maximum values in parenthesis are indicated above the scatters of each cell population. A negative correlation was found between IFN-gamma mRNA expression and severity rank order (Dukes' stage A<B<C) (r = - 0.501, p = 0.039). P value on the figure indicates a significant difference between the groups of patients with Dukes' stage A/B (median value: 463.5) *versus* C.

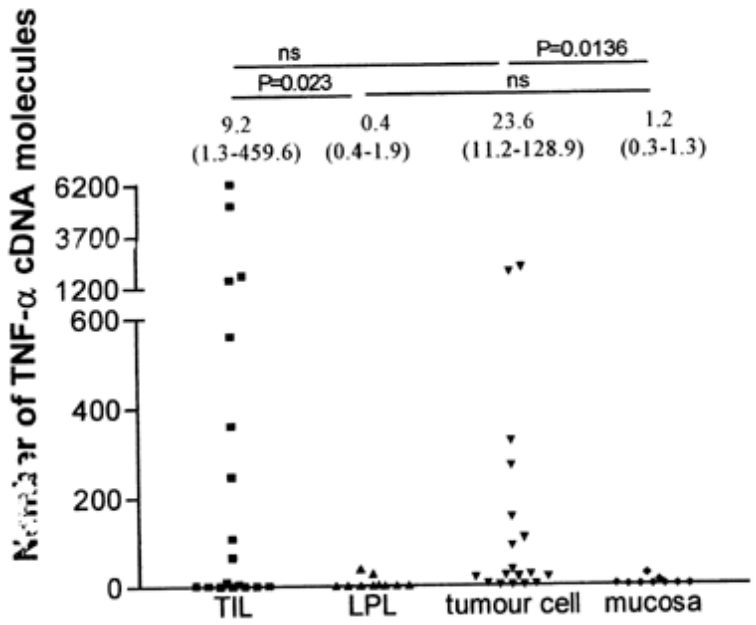


Figure 3

TNF-alpha mRNA in cells from tumour and normal colon mucosa.

TNF-alpha transcripts were quantified by semiquantitative RT-PCR using pQA1 competitor DNA in TIL (n = 19), tumour cells (n = 18), lamina propria-infiltrating leukocytes (LPL) (n = 10) and epithelial cells in normal mucosa (n = 9) from patients with colorectal carcinoma (see legend to Figure 1). Values are numbers of TNF-alpha cDNA/10^6 beta-actin cDNA molecules.

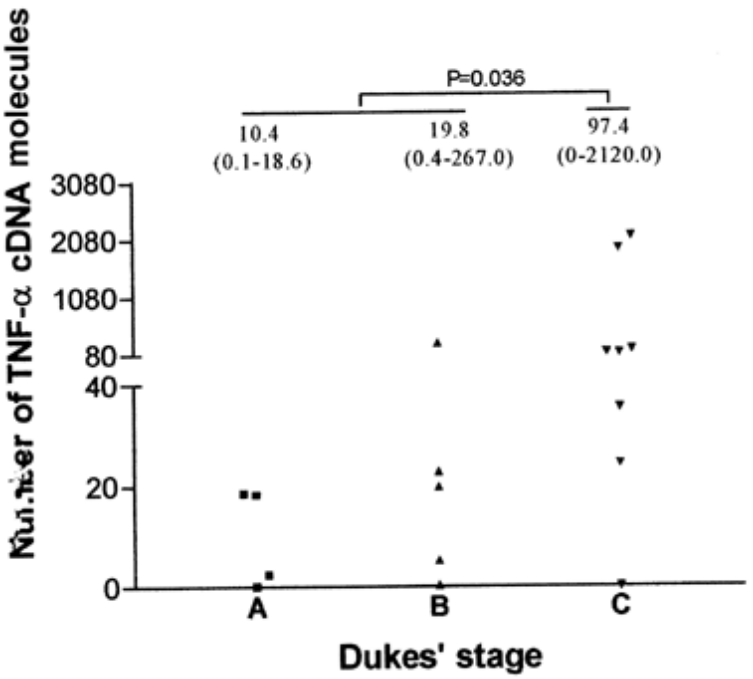


Figure 4

TNF-alpha mRNA expression in tumour cells from different stages of colorectal cancer.

Amplified cDNA were quantified by competitive PCR in tumour cells from patients with Dukes' A (n = 4), B (n = 5) and C (n = 8) stages of colorectal carcinoma as described in Materials and Methods. Values are numbers of TNF-alpha cDNA/10^6 beta-actin cDNA molecules. Median numbers of TNF-alpha specific DNA amplicons/10^6 beta-actin DNA amplicons with the minimum and maximum values in parenthesis are indicated above the scatters of each cell population. TNF-alpha mRNA correlated positively with severity rank order (Dukes' stage A<B<C) (r = 0.5800, p = 0.0147). P value on the figure indicates a significant difference between the groups of patients with Dukes' stage A/B (median value: 18.26) *versus* C.

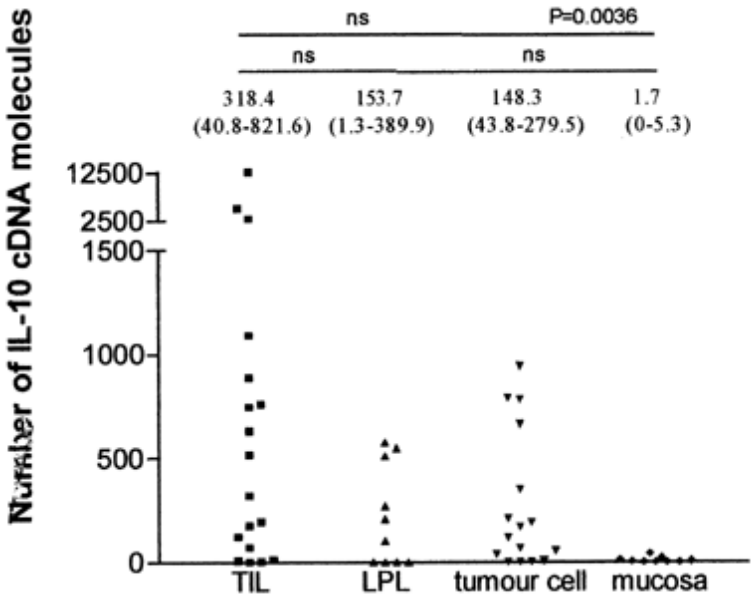


Figure 5

IL-10 mRNA in cells from tumour and normal colon mucosa.

IL-10 transcripts were quantified by semiquantitative RT-PCR using pQB3 competitor DNA in TIL (n = 19), tumour cells (n = 18), lamina propria-infiltrating leukocytes (LPL) (n = 10) and epithelial cells in normal mucosa (n = 9) from patients with colorectal carcinoma (see legend to Figure 1). Values are numbers of IL-10 cDNA/10^6 beta-actin cDNA molecules.

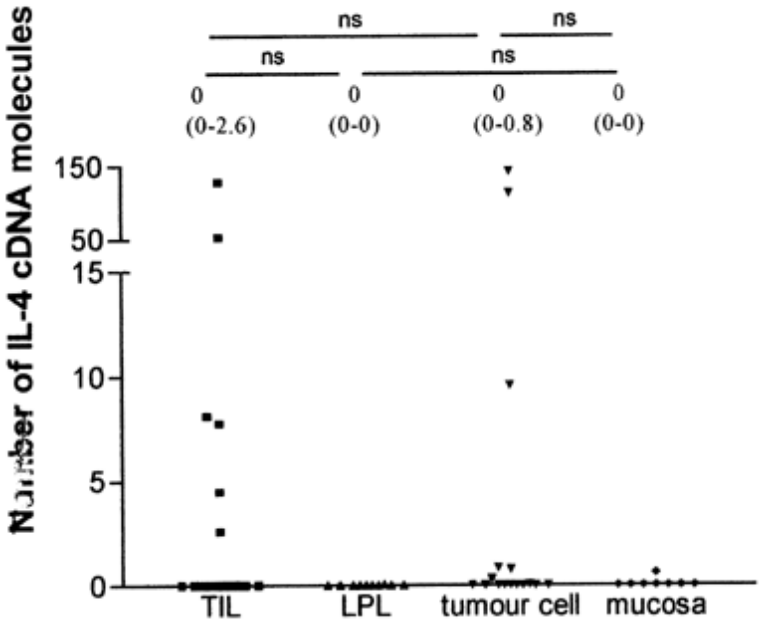


Figure 6

IL-4 mRNA in cells from tumour and normal colon mucosa.

IL-4 transcripts were quantified by semiquantitative RT-PCR using pQA1 competitor DNA in TIL (n = 17), tumour cells (n = 16), lamina propria-infiltrating leukocytes (LPL) (n = 10) and epithelial cells in normal mucosa (n = 9) from patients with colorectal carcinoma (see legend to Figure 1). Values are numbers of IL-4 cDNA/10^6 beta-actin cDNA molecules.

Table 1
Clinical profiles of patients with primary colorectal adenocarcinoma

Patient	Age (yr)	Sex	Metastasis	Dukes' stage	Diameter of the tumour (cm)
1	65	f	no	B	6.0
2	69	m	no	A	6.0
3	55	m	no	B	4.0
4	76	m	yes	C	6.0
5	83	m	yes	C	4.0
6	76	f	no	B	4.0
7	69	m	yes	C	9.0
8	54	m	no	A	2.5
9	80	m	yes	C	6.0
10	75	f	–	–	–
11	60	f	yes	C	3.0
12	77	f	no	B	–
13	70	f	yes	C	6.9
14	89	m	no	B	3.5
15	20	f	no	A	6.0
16	77	f	no	B	2.5
17	80	f	yes	C	8.0
18	72	m	yes	C	7.0
19	42	m	no	A	7.0
20	75	f	no	B	4.0
21	74	f	no	A	8.0

f: female, m: male; 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 metastatases.

Table 2
Oligonucleotide primers

Cytokine	Sense primer 5' 3'	Antisense primer 5' 3'	Amplicon[1] length (bp)	Competitor DNA specific 5'-biotinylated oligonucleotide 5' 3'	cDNA-specific 5'-biotinylated oligonucleotide 5' 3'
IFN-gamma	GCAGAGCCAA ATTGTCTCCT	ATGCTCTTCG ACCTCGAAAC	290	AGACCCCAGCA GAGAATGGAA	GATGACCAGAGC ATCCAAAAGAG
IL-4	TGCCTCCAAG AACACAACTG	AACGTACTCT GGTTGGCTTC	224	AGACCCCAGCA GAGAATGGAA	AGGGCTGCGA CTGTGCTCCG
IL-10[2]	ATGCTTCGAG ATCTCCGAGA	AAATCGATGA CAGCGCCGTA	269	GGATCCTTTG CATCGACTGC	GGAGGACTTT AAGGGTTACC
TNF-alpha	ACAAGCCTGT AGCCCATGTT	AAAGTAGACC TGCCCAGACT	427	AGACCCCAGCA GAGAATGGAA	CCTCTTCAAGG GCCAAGGCTGC
beta-actin[2]	GGGTCAGAAG GATTCCTATG	GGTCTCAAAC ATGATCTGGG	237	GTGAGGGACAT GCTCACGCAGC	CGACGAGGCCC AGAGCAAGAGA

[1]IFN-gamma, IL-4, TNF-alpha PCR gives a standard amplicon of 369-371 bp on pQA-1 competitor plasmid, beta-actin and IL-10 PCR results in a 399 and a 407 bp amplicon on pQB-3 competitor DNA, respectively.
[2] IL-10 and beta-actin primers are in pQB-3.

Authors

A. Csiszar, T. Szentes, B. Haraszti, W. Zou, D. Emilie, G. Petranyi, E. Pocsik

National Institute of Haematology and Immunology, Daróczi út 24, Budapest, Hungary H-1113.

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IL-10, recombinant, rat, cytokines.

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