Cytokine Expression in Colon Carcinoma

PETER K. BAIER, GUIDO WOLFF-VORBECK, STEPHAN EGGSTEIN, ULRICH BAUMGARTNER and ULRICH T. HOPT

University of Freiburg, Department of Surgery, Hugstetterstraße 55, D-79106 Freiburg, Germany

Abstract. Background: Cytokines reflect the activity of the immune system. We analyzed the local expression of characteristic cytokines indicating the level of activity of unspecific inflammatory cells, Th1-cells and Th2-cells in colon cancer. Materials and Methods: In 25 tumor/mucosa pairs, IL-1α, IL-2, IL-4, IL-5, IL-15, TNF-α and IFN-γ were measured by real-time PCR. Results: There was a significant increase in IL-1α, IL-4, IL-5 and TNF-α and a significant decrease in IL-2 in tumor tissue compared to normal mucosa. Discussion: The cytokine profile in colon cancer indicates a strong unspecific inflammatory reaction in the tumor tissue represented by high levels of IL-1 and TNF-α. The comparatively low level of IL-2 suggests suppression of a specific immunological reaction, namely Th1-cells. It can be hypothesized that this is a result and/or cause of local immune escape mechanisms. Furthermore, there is an activation of Th2-cells in the carcinomas.

Cancer cells express antigens to which the immune system responds with a cytotoxic T lymphocyte (CTL) infiltration and with a delayed type hypersensitivity (DTH) reaction (1, 2); the same holds true in colorectal cancer cells (3). The immune system controls the development of malignancies. If affected, the incidence of malignancies rises, as can be observed in immunosuppressed patients after organ transplantation. The development of a clinically manifest carcinoma implies that immunological control has been insufficient. In this context, many systemic and local immune escape mechanisms are being discussed (1, 4). To determine the activity of unspecific immunity, Th1-cells and Th2-cells and possible immune escape mechanisms in colon cancer, we analyzed the expression of various cytokines involved in the activation and differentiation of macrophages and T lymphocytes (5). We compared the level of expression of IL-1α, IL-2, IL-4, IL-5, IL-15, TNF-α and IFN-γ in colon carcinoma tissue to normal colon mucosa by real-time PCR in 25 sample pairs. PCR analysis of cytokine expression provides a highly sensitive means of quantifying the antigen-specific immune response(6, 7). As an internal control, we analyzed the expression of FAS-ligand, well known to be expressed more strongly in carcinoma cells than in mucosa (8).

Materials and Methods

Specimens. With the consent of the local ethics board, paired samples of colorectal mucosa and adenocarcinomas derived from 25 patients, who were operated on for colorectal carcinoma in the Department of Surgery, University of Freiburg, Germany, were investigated. Tumor samples were taken from vital areas of histopathologically confirmed carcinomas. Mucosa samples were derived from unaffected mucosa 2 cm distal to the oral resection margin by sharp dissection from the submucosa. The tissues were harvested immediately after resection of the colon, washed in ice-cold phosphate-buffered saline and snap-frozen in liquid nitrogen.

RNA preparation. Preparation of the RNA was performed with the total-RNA easy mini prep kit (Qiagen, Hilden, Germany) with a DNAse step, according to the instructions of the producer.

Real-time PCR. The real-time PCR was performed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Weiterstadt, Germany) using random hexamers (500ng t-RNA in 34.75 μl + 65.25 μl RT mix; 10 min 25°C, 60 min 48°C; 5 min 95°C) and Pre Developed TaqMan Assay Reagents (Applied Biosystems) in a two-step technique according to the manual of Applied Biosystems. As a control gene 18s-rRNA was used in each PCR. (2.5 μl Cytokin Target Mix, 2.5 μl Ribosom Control Mix, 25 μl Master Mix, 20 μl probe; 10 min 95°C, 40X (15 sec 95°C, 1 min 60°C)) PCR was performed in ABIPrism7700SequenceDetectionSystem (Applied Biosystems).

Statistical and data presentation. Comparative C_T method for separate tubes: from the real-time amplification plot, the threshold cycle for target amplification was calculated: C_T. For each specimen and cytokine the difference between the C_T value of the 18s-RNA and cytokine RNA was calculated: ΔC_T. Since in every probe there was more 18s-rRNA than cytokine RNA, a low ΔC_T value indicates a large amount of cytokine RNA.
The $\Delta C_t$ values of the tumor and corresponding mucosa were compared by a $t$-test for paired values. The values are shown as MEAN ± SEM. The graphs show boxes and whiskers. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile). Whiskers extend down to the smallest value and up to the largest. In the cases of IL-4 and IL-5, where specific cytokine RNA was not detectable in all specimens, we used the contingency tables and the Chi-square test with the values "detectable" and "not detectable". The data are shown in scatter graphs.

Results

Fas-ligand RNA was more often detectable in tumor than in mucosa tissue: $\Delta C_t = 24.31 ± 0.2651$ in tumor and $\Delta C_t = 26.09 ± 0.2336$ in mucosa; $p < 0.0001$.

For IL-1α we found a $\Delta C_t = 25.31 ± 0.2774$ in the mucosa and a $\Delta C_t = 20.88 ± 0.3684$ in the tumor tissue, respectively. Since the difference ($\Delta C_t$) of the $C_t$ from IL-1 and 18s-rRNA was smaller in tumor than in mucosa tissue, there was more IL-1 RNA in the tumor than in mucosa. The difference is significant: $p < 0.0001$ (Figure 1).

The same could be observed for TNF-α (Figure 2): in mucosa $\Delta C_t = 21.44 ± 0.2672$ and in the tumor $\Delta C_t = 20.46 ± 0.2091$. There was significantly more TNF-α RNA in the tumor than in mucosa tissue; $p = 0.0059$.

In the case of IL-2, the contrary could be shown (Figure 3): $\Delta C_t$ in mucosa was $22.93 ± 0.2594$ and in the tumor $26.99 ± 0.2496$. There was significantly more IL-2 RNA in the mucosa $p < 0.0001$.

With respect to INF-γ and IL-15, we did not find a significant difference between the tumor and normal mucosa with $p$ values of $p = 0.53$ (Figure 4) and $p = 0.35$ (Figure 5), respectively.

Due to non-detectable values of IL-4 and IL-5 (levels below sensitivity of PCR), only 11 and 7 pairs were available for investigation. Therefore, we used the contingency tables and the Chi-square test with the categories "detectable" and "not detectable". IL-4 and IL-5 were more often detectable in the tumor than in the mucosa ($p = 0.001$ and $p = 0.0012$, respectively). Correspondingly, Figures 6 and 7 are given as scatter graphs with a marked mean of the detectable values.

Discussion

Former studies tried to correlate the quantity of leukocyte infiltration in colon cancer and prognosis(9, 10). Yet, these
studies analyzed the morphology of the infiltrating cells and were unable to differentiate between quiescent and active leukocytes. There is still a debate on the relevance of infiltrating leukocytes (11). One way to focus more on cell function than morphology is to analyze cytokines representing activated leukocytes by real-time PCR. This approach was used successfully to monitor the cellular immune response in several tumor immunotherapy studies (12, 13).

In this study, real-time PCR was used for the first time as a tool for analyzing natural immunity in colon cancer. Healthy mucosa of the same donor was used as a reference, since it is one of the body’s active immunological tissues with physiological levels of cytokines. Thus, the intraindividual cytokine expression in malignant tissue and healthy mucosa was studied.

It is well known that, due to the use of homogenates of tissue, the specific cell type of cytokine production cannot be determined. Yet, the difference between cytokine expression in normal mucosa and malignant tissue can clearly be attributed to the host reaction to malignancy.

We chose to investigate the various mRNA values instead of cytokine proteins because protein levels of cytokines in a specimen may not represent the tissue alone, but also the status of the whole body. This is especially relevant during
an operation where the altered plasma levels could influence the level of the specific cytokine (14). Therefore, we analyzed mRNA since it is clear that this reflects the local cytokine expression.

The significantly higher expression of FAS-ligand in tumor cells is not a new finding. We could reproduce these results, which implies that this method is capable of distinguishing between different levels of expressions of a gene in tumor and mucosa tissue.

We found a significant increase in IL-1 and TNF-α in the tumor specimens. This is not surprising since a malignant lesion is linked to inflammation (15) and both cytokines are expressed by activated macrophages during mucosal infections (16). Therefore, in a colon carcinoma there is a stronger activation of the unspecific immunosystem than in normal mucosa.

Cytokines can help elucidate further aspects of the specific immune response, for example, the proliferation and differentiation of activated T<sub>H</sub>1-cells. They are regulated by IL-2 and INF-γ. Here, we found a significant decrease in the IL-2 mRNA levels in the tumors. A recent study analyzing peritoneal washing fluid from gastric cancer patients by PCR demonstrated a comparable result with a decrease of IL-2 in patients with peritoneal seeding (17).

There are several mechanisms known to reduce IL-2 expression. In this case, a lack of the costimulation (18, 19), calcineurin inhibition (20, 21) or, more probably, the consequence of CD4+ CD25 bright cells with regulatory T activity (22, 23), may be discussed. The impaired IL-2 expression may reflect an immune escape mechanism. At least two mechanisms could plausibly explain this phenomenon: antigen presentation without costimulation leading to T-cell anergy and tolerance, or an increased activity of regulatory CD4+/CD25 bright cells.

The amount of INF-γ showed no significant difference between the tumor and mucosa, so we could not demonstrate an immune escape mechanism related to this T<sub>H</sub>1 cytokine.

As characteristic cytokines for T<sub>H</sub>2 cells, we analyzed IL-4 and IL-5. IL-4 and IL-5 are more often detectable in tumors than in mucosa specimens, indicating an activation of T<sub>H</sub>2 cells in the tumor.

The demonstrated decrease in IL-2 is a local mechanism possibly explaining an immune escape in colon carcinomas. Based on this finding, research focusing on T<sub>H</sub>1 activity is warranted. Furthermore, investigations will be necessary to help explain the cause of increased T<sub>H</sub>2 activity (e.g.: humoral anti-tumor vs. anti-bacterial stimulation) and the relevance of IL-15 in tumor tissue. We know that the demonstrated decrease in IL-2 mRNA is only a first step. Further evaluation is necessary by analyzing IL-2 protein expression and by analyzing the mechanisms leading to the decreased IL-2 in tumors.

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