IL-6 AND IL-8 RESPONSES OF COLORECTAL CANCER IN VIVO AND IN VITRO
CANCER CELLS SUBJECTED TO SIMVASTATIN

Recent investigations suggest that proinflammatory cytokines such as IL-6 and IL-8 are involved in the development of colorectal cancer (CRC), whereas statins, primarily used to decrease high levels of blood cholesterol, exhibit pleiotropic effects on carcinogenesis. In the present study we compared the expression of IL-6 and IL-8 in tissue samples of tumor and adjacent normal colon mucosa obtained from patients with advanced colorectal cancer (CRC). The analysis of mRNA expression for these proinflammatory cytokines determined by RT-PCR showed a higher level of IL-8 mRNA in tumor tissue than in normal mucosa, while IL-6 was similarly expressed in tumor and normal tissue. The mean values of serum levels of both IL-6 and IL-8 were significantly higher in CRC patients than in healthy volunteers. Surgical removal of the tumor resulted in a prompt decrease of serum level of IL-8 already on the third day, whereas IL-6 level was transiently increased to become lower only after 7-10 days. Treatment of CRC with simvastatin (80 mg/day for 14 days) led to a significant decrease of serum IL-6, while the IL-8 level was less affected. The in vitro experiments on colorectal cancer-derived cell lines (HT-29 and Caco-2) demonstrated that application of simvastatin decreased generation of both IL-6 and IL-8. The differences in response of serum levels of IL-6 and IL-8 after tumor removal and treatment with simvastatin are novel observations suggesting distinct pathological roles of the two cytokines in CRC development. We conclude that 1) colorectal carcinogenesis is accompanied by increased synthesis and release of proinflammatory cytokines such as IL-6 and IL-8; 2) simvastatin therapy results in a decrease in serum level of proinflammatory cytokines, especially IL-6 in CRC and 3) simvastatin inhibits release of IL-8 and IL-6 from colorectal cell lines.

Key words: colorectal cancer, statins, IL-6, IL-8, tumor, caco-2 cells, tumor necrosis factor alpha

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of mortality and accounts for approximately 200000 deaths per year in Europe and the USA (1). The molecular mechanism of colorectal carcinogenesis is still poorly understood as it is a multi-step process in which several different factors are involved. Recent publications drew attention to interleukin-6 (IL-6) in CRC pathogenesis. Interleukin-6 is a pleiotropic cytokine with a wide range of biological effects including inflammatory, autoimmune and cancerogenic (2). Previously, it was demonstrated that serum level of IL-6 is increased in CRC patients (3-5) and that it correlates with tumor size (6). Numerous studies have already documented anti-inflammatory properties of statins, used primarily as inhibitors of HMG-CoA reductase - a key enzyme in cholesterol synthesis (21). The mechanism of anti-inflammatory activity of statins became clear after finding that simvastatin or fluvastatin blocks the presence of IL-6 and this effect was mediated by Bcl-2 (13). On the basis of these findings a new therapeutic approach was proposed by Naka et al. (2) to block the IL-6 signal using humanized anti-IL-6 antibody as in treatment of rheumatoid arthritis, Castleman's disease and multiple myeloma.

Besides IL-6, also IL-8, is a well known proinflammatory cytokine and important chemoattractant factor for leukocytes. Moreover, it was shown that IL-8 contributes to cancer progression through its potential functions as a mitogenic, motogenic and angiogenic factor (15). Additionally, an increase in serum and cancer tissue IL-8 levels was demonstrated in CRC patients (10, 16). The expression of IL-8 in tumor tissue significantly correlated with tumor size, depth of infiltration and liver metastasis (17), as well as with tumor stage (18). Recombinant human IL-8 was reported to enhance the in vitro proliferation of CRC cells (19). Kullman et al. (20) demonstrated that the transcription factor C/EBPbeta (CCAAT-enhancer-binding protein) cooperates with IL-6 to amplify the activation of the inflammatory network, including IL-8.

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the synthesis of IL-6 and IL-8 in isolated blood monocytes that had been stimulated by C-reactive protein or LPS (22-24). Sakoda et al. (25) showed, that simvastatin dose-dependently diminished IL-1α-induced IL-6 and IL-8 production in human epithelial cells. Similar effects were observed in fibroblast-like synoviocytes (FLS). In this cell line, statin inhibited IL-1-induced NF-κB activation (26).

Besides the anti-inflammatory effect, increasing clinical evidence suggests that statins, independent of their effects on serum cholesterol level, may also play a potential role in prevention and treatment of cancer (27). Extensive studies over the last four years have demonstrated that statins generate pro-apoptotic, tumor growth inhibitory and pro-differentiation responses of neoplastic cells of diverse origin (28, 29).

Until now, an information about the effect of statins on the production of IL-6 and IL-8 in CRC cells in vitro and the effect of statin treatment of CRC is very limited. The aim of the present study here was; (1) to compare the expression of IL-6 and IL-8 in normal colonic mucosa, (2) to determine the in vitro effects of simvastatin treatment and surgical tumor removal on serum levels of IL-6 and IL-8 in patients with CRC and (3) to investigate the in vitro effect of simvastatin on protein IL-6 and IL-8 expression in CRC cell lines.

MATERIALS AND METHODS

**In vivo studies on CRC patients**

25 patients with CRC diagnosed by endoscopy and histology of biopsy samples and 25 healthy volunteers were included in the present study (30). From each patient and healthy volunteer blood samples for measurement of cytokines were obtained. In addition, from 6 patients tumor tissue samples, as well as normal colon mucosa, were collected during surgical removal of the tumor. The samples were immediately frozen in liquid nitrogen and kept at -80°C for further analysis. From group of 16 CRC patients the blood samples were collected immediately before curative surgery and 8 days after operation. Finally, the group of 9 CRC patients was treated with simvastatin (daily dose of 80 mg) for 14 days before operation, and blood samples were collected at the start and after 14 days of therapy.

All patients originated from an urban area of Kraków (Poland). The study was approved by the Ethical Committee at the Jagiellonian University Medical College and the informed consent was obtained from all patients included into this study.

**In vitro studies on CRC cells**

Two cell lines derived from colon carcinoma; Caco-2 and HT-29 (American Type Culture Collection) were used. Caco-2 cells were cultured in MEM (Minimum Essential Medium Eagle) supplemented with non-essential amino acids, 2 mM glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum (FBS) at 37°C in the atmosphere of 95% air and 5% CO₂. All reagents were obtained from Sigma (Germany), unless otherwise stated. At the beginning of experiment, approximately 25000 cells/cm² were seeded on 24-well plates and cultured for 48 h. Then, the medium was replaced by the medium containing 5% of FBS and indicated concentration of simvastatin (SV) (Sigma), and the cells were further cultured for 48 h with medium replaced every 24 h.

HT-29 cells were cultured in high glucose DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS at 37°C in the atmosphere of 95% air and 5% CO₂. Approximately 25000 cells/cm² were seeded on 24-well plates and cultured for 48 h. Then the medium was replaced by serum free medium containing indicated concentration of simvastatin. The cells were further cultured for 24 h or 48 h.

**RNA extraction and RT-PCR**

Total RNA was prepared from cell culture or tissue samples using TRIZOL or GTC reagents according to vendor's protocol. 2 µg of total RNA was used to synthesize the first strand of cDNA using MMLV Reverse Transcriptase 200 µg (Promega), 0.5 mM dNTP (Fermentas), 0.5 µg Oligo (dT) Primer as described before (31). For determination of IL-6 mRNA expression the following primers were used: forward primer; 5'-CCTAAAAGCTGCCGAGAATTG-3' and reverse primer; 5'-ATTCAATGAGGAGACTTGCC-3'. The PCR program was following: denaturation at 94°C for 4 min followed by 35 cycles; 94°C for 45 s, 56°C for 45 s, 72°C for 120 s and finally a single stage of 72°C for 5 min (product length=284 bp).

For determination of IL-8 mRNA expression the following primers were used: forward primer; 5'-CTCTCTCGGCAGCC TTCTGTA-3' and reverse primer; 5'-CCCTTGACCCAGTT TTCTCTT-3'. The PCR program was following: denaturation at 95°C for 4 min followed by 35 cycles; 95°C for 45 s, 65°C for 45 s, 72°C for 50 s, and finally a single stage of 72°C for 5 min (product length = 240 bp).

For determination of EF 2 (translation elongation factor 2) mRNA (house-keeping gene) expression the following primers were used: forward primer; 5'-GACATCACCAAGGGTGTGC AG-3' and reverse primer; 5'-GCG GTC AGC ACA ATG GCA TA-3'. The PCR program was following: denaturation at 95°C for 120 s followed by 28 cycles; 92°C for 30 s, 58°C for 45 s, 72°C for 90 s, and finally a single stage of 72°C for 5 min (product length = 218 bp).

The PCR products were separated in agarose gel electrophoresis and the abundance of cDNA in each sample was estimated by video densitometry analysis (Imager BIORAD) using the Quantity program, and expressed as a ratio of the tested cytokine cDNA versus the calculated intensity to EF 2 product used as a reference gene.

**Measurement of IL-6 and IL-8 in serum**

The levels of IL-6 and IL-8 in serum and tissue homogenates were determined using a specific ELISA kits (Quantikine R&D Systems) according to vendor's protocol.

**Statistical analysis**

All values are presented as a mean ± SEM. Statistical significance was evaluated by paired t-test.

**RESULTS**

Figs 1A and 1B show representative mRNA expression for the proinflammatory cytokines IL-8 and IL-6 expressed in tissue samples obtained from three patients with CRC. Similar results with mRNA expression of IL-8 and IL-6 were recorded in remaining tested samples of CRC but these results have been omitted for the sake of clarity. In case of IL-8, normal mucosa contains only traces of corresponding mRNA and, therefore, an increase observed in tumor tissue is statistically significant (p<0.05). On the other hand, IL-6 mRNA is commonly expressed in both tumor and normal colonic mucosa of CRC patients, and only a slight and non-significant tendency to up-regulation of IL-6 gene expression in the tumor tissue was observed (Fig. 1B).
Both cytokines were present in the sera of all tested CRC patients. Their levels in CRC patients, determined by ELISA, were significantly higher than those observed in age-matched healthy volunteers, reaching the mean values of 29.3 pg/ml for IL-8 and 31.4 pg/ml for IL-6, respectively (Fig. 1C and D).

Surgical resection of CRC resulted in a strong reduction in serum IL-8 concentration (Fig. 2A). The decrease was clearly visible already on the third day after the surgery. On the other hand, the serum level of IL-6 was transiently increased in some patients during the first 2-3 days after surgical removal of the tumor (Fig. 2B). These opposite changes of IL-8 and IL-6 levels in the blood of patients following removal of the tumor probably reflect the differences in the origin and biological role of these two cytokines in CRC.
well as the attenuation of the TNF-α-induced expression of IL-6 in HT-29 cells. In our experiments, HT-29 cells failed to produce IL-6 in response to TNF-α, in contrast to Caco-2 cells (Fig. 4). The mechanism of IL-6 up-regulation in the serum of CRC patients is not fully understood but some data suggest that IL-6 signaling promotes tumor growth in CRC (1) and diminishes T-cell stimulation by TGF-β that may lead to increased IL-6 production by T cells (33). Pretreatment of Caco-2 BBE cell line with TGFβ was associated with a down-regulation of IL-6-induced tyrosine phosphorylation of STAT1 and STAT3 (34).

Little information is available regarding the mechanism of IL-8 increase in the serum of CRC patients. Our study showed significantly higher serum level of IL-8 in CRC patients in comparison to healthy controls, and stronger expression of IL-8 in tumor tissue (compared to intact - non tumorous adjacent colon mucosa). This observation is in agreement with the study by Rubie et al. (18) also showing that IL-8 mRNA and protein expressions were significantly higher in CRC tumor tissue when compared to the neighboring tissues. We have found a high IL-8 production not only in the tumor of CRC patients but also a high constitutive synthesis of IL-8 in human colorectal cancer cells line (HT-29). According to Dixon et al. (35) in HT-29 cell line IL-8 mRNA is transcribed constitutively and shows a long half-life. The observed mRNA stabilization may result from defective recognition of regulatory sequences ARE in the un-translated regions at the 3'end of mRNA (IL-8 3'UTRs contain class II ARE). A protein known as HuR stabilization factor which binds ARE is over-expressed in tumors. By binding to ARE HuR may up-regulate synthesis of IL-8 (36).

The tumor removal resulted in the reduction in serum IL-6 and IL-8 levels, but with a strikingly different kinetics: IL-8 decrease was prompt and clearly visible already after 3 days (Fig. 2A), whereas at this time IL-6 level was increased in some patients and significant reduction was noted only after 7-10 days (Fig. 2B). This could be explained by the fact that the cells of CRC themselves are the main source of serum IL-8 so tumor removal leads to direct reduction of IL-8 in serum, whereas in case of IL-6 serum cytokine derives also from cells of the immune system, which is stimulated and remains active even after surgical operation. Only after prolonged period of time (10 days after operation), when operation-induced injury and inflammation was resolving, the serum IL-6 decreased, but was still above the level found in healthy subjects. Our observations are supported by the data of other authors who found increased levels of IL-6 at 24 h after operation (5) followed by a decrease only after 16 days (3) or even after 3 months (6).

In patients treated for 14 days with simvastatin (80 mg/day) prior to the surgery we observed significant decrease in IL-6 serum concentration, while the decrease in the serum IL-8 levels were less pronounced and not significant (Fig. 3). The treatment with simvastatin was well tolerated by all treated subjects and no adverse events were recorded in any of 9 CRC patients subjected to 14 days of simvastatin therapy.

To clarify the mechanism responsible for the decrease of serum cytokine level by simvastatin we carried out the in vitro experiments on cell lines derived from CRC (HT-29 and Caco-2). The in vitro analysis showed that IL-8 is constitutively generated by HT-29 cell line, whereas the synthesis of IL-6 requires the stimulation of Caco-2 cells with another proinflammatory cytokine such as TNF-α to respond to the simvastatin action. As shown in Fig. 4, only higher concentrations of simvastatin in vitro caused a decrease in expression of IL-8 in HT-29 cells, as well as the attenuation of the TNFα-induced expression of IL-6 in Caco-2 cells. It should be added, however, that the most effective simvastatin concentration exceeded 5 µM, i.e. the level which is unlikely to be achieved in the in vivo studies.

**DISCUSSION**

Interleukin-6 is known to participate in the immune response or tumor metastasis, and to induce fever and other symptoms of acute phase reaction, such as synthesis of specific proteins in the liver (2), whereas IL-8 is regarded as a typical chemokine, being potent chemoattractant and angiogenic factor (15). In this study we found that genes coding for these two proinflammatory cytokines of different functions, IL-6 and IL-8, are expressed in the CRC cells. In addition, we demonstrated that the cells derived from the CRC, such as HT-29 and Caco-2 cells, are capable to produce IL-6 and IL-8 (see Fig. 4). Thus, it can be concluded that growing colorectal tumor produces these cytokines and may contribute to their increased plasma levels observed in CRC patients, and to influence various aspects of inflammatory reaction accompanying cancer progression. This explains the fact that the CRC carcinogenesis is accompanied by inflammatory symptoms as described previously (4-6, 11, 18).

In agreement with previous observations we found that CRC patients exhibit significantly higher serum levels of IL-6 (3-6) and IL-8 (5, 18, 19) in comparison to healthy volunteers. However, this fact does not necessarily imply that tumor cells are the only source of plasma IL-8 and IL-6 since other cells of leukocytic or monocytic origin infiltrating the growing tumor may also contribute to the generation of these cytokines (2). It should be remembered that the production and secretion of cytokines depend not only on the cell type but also on their differentiation stage.

Brozek et al. (32) noticed, that IL-6 mRNA and protein expression were low in moderately and well differentiated Caco-2/AQ and COKA-1A cell lines, but high in poorly differentiated COKA-13 cell line treated with IL-1β. In our experiments, HT-29 cells failed to produce IL-6 in response to TNF-α, in contrast to Caco-2 cells (Fig. 4). The mechanism of IL-6 up-regulation in the serum of CRC patients is not fully understood but some data suggest that IL-6 signaling promotes tumor growth in CRC (1) and diminishes T-cell stimulation by TGF-β that may lead to increased IL-6 production by T cells (33). Pretreatment of Caco-2 BBE cell line with TGFβ was associated with a down-regulation of IL-6-induced tyrosine phosphorylation of STAT1 and STAT3 (34).

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Very important observation of the present study was the inhibitory effect of statins (simvastatin) on serum level of proinflammatory cytokine IL-6 (at the dose of 80 mg/day for 14 days) (Fig. 3). Whether this therapy applied prior to operation may have general beneficial effects and facilitate faster recovery should be verified in a large scale clinical trial. The decrease of serum level of IL-8 after simvastatin treatment was visible but not significant. It is difficult to explain the mechanism of simvastatin-induced reduction of cytokine expression in CRC patients and observed differences between IL-6 and IL-8 response. As indicated by results of our experiments based on determination of mRNAs coding for these cytokines, and by the results of other authors, simvastatin acts at the pretranslational level of cytokine gene expression but the detailed mechanism requires further studies. Several large clinical trials showed that statins can reduce the risk of colorectal cancer. Poynter et al. (37) showed that the use of statins for at least 5 years was associated with a relative reduction in risk of CRC. Siddiqui et al. (38) reported recently that overall 5-year survival in statin users (37%) was slightly lower than in statin non-users (33%) but, most important, the long-term use of statin in former patients was associated with less advanced tumor stage, lower frequency of distant metastases and generally much better clinical outcome. Interestingly, Hofmeister et al. reported that the combination of statins and low dose aspirin causes a significant reduction in risk for CRC (39). In contrast to these reports, the recent meta-analysis of 18 studies (40) involving more than 1.5 million patients showed only modest reduction in the risk for CRC by statins. However, the authors could not rule out the possibility that the higher doses of statins are needed to induce stronger chemo-preventive effect on the colorectal carcinogenesis. Further limitation of this study was that the treatment and follow up times only lasted for an average of 5.9 years, which could be too short period to draw definite conclusions.

In summary, our study demonstrated that 1) colorectal carcinogenesis is accompanied by increased synthesis and release of proinflammatory cytokines such as IL-6 and IL-8; 2) statin therapy results in a decrease in serum level of proinflammatory cytokines, especially IL-6 in CRC patients, and 3) statin inhibits release of IL-8 and IL-6 directly from the colorectal cell lines.

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