Impact of COX-2, IL-1β, TNF-α, IL-4 and IL-10
on the process of carcinogenesis in the large bowel

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In recent years there have been many intensive studies on the molecular mechanisms involving the carcinogenesis of colorectal cancer (CRC). An inflammatory process and genetics play the key role in neoplasia of CRC. Currently, there are two known pathways of CRC carcinogenesis, such as the adenoma and the serrated adenoma, which are referred to as "classic" and "alternative", respectively. Among all the components of the inflammatory process, the proinflammatory and anti-inflammatory cytokines play a major role as a factor influencing the process of malignant transformation. In our study we focused on key inflammatory factors such as cytokines interleukin (IL)-10, IL-1β, IL-4, tumor necrosis factor α (TNF-α) and cyclooxygenase-2 (COX-2) in adenomas, serrated adenomas, hyperplastic polyps, adenocarcinomas and normal mucosa. Our study confirmed the hypothesis that inflammation has a major effect on carcinogenesis of CRC. Our studies also showed the difference in carcinogenesis of CRC. It showed a greater effect of the inflammatory process in carcinogenesis of CRC by a "serrated" (alternative) way as compared to the classic way. In a serrated way all the inflammatory factors had a higher expression. It might suggest that effectiveness of cancer prevention with the use of NSAIDs has a greater impact in patients whose tumors were formed in an alternative way. Additionally, it also showed that the inflammatory process has no influence on the final form of cancer.

Key words: colorectal carcinogenesis, serrated pathway, inflammatory cytokines.

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world. Moreover, among women, it became the second one in the world according to statistics from 2008. Each year, there is an alarming increase in the number of cases and mortality from CRC. Many studies and collected statistical data predict that the upward trend of incidence and mortality will continue in the next few years. The highest incidence rate of CRC occurs in the countries of Western Europe and North America, an area recognized as highly developed nations. A sedentary lifestyle and a poor quality diet which is characteristic of these countries contribute to the carcinogenesis of CRC.

Based on several studies conducted around the world it had been proven that a diet rich in fats and low in cellulose and natural vitamins increases the risk of CRC [1]. A high mortality rate is associated with delayed diagnosis and lack of prevention. Unfortunately, there is also a global lack of public awareness about this threatening phenomenon. However, in recent years there have been many intensive studies on the molecular mechanisms involving the carcinogenesis of CRC. An inflammatory process and genetics play the key role in neoplasia of CRC. Currently, there are two known pathways of CRC carcinogenesis, such as the adenoma and the serrated adenoma, which are referred to as "classic" and "alternative", respectively. The mechanism that occurs in the classic

way is responsible for about 80% of CRC. The key role in this mechanism is played by mutations of APC, Wnt/β-catenin, p53 and K-ras genes. The alternative CRC, which is described in approximately 15% of sporadic colorectal cancers, is due to excessive methylation of promoter sites of proteins, especially proteins that play a role in the control of the cell cycle and DNA repair mechanisms (hMLH-1) mutations in the BRAF [2]. The recently intensively studied factor which influences the CRC carcinogenesis is an inflammatory process. This is probably one of the most important factors in addition to genetic abnormalities. The inflammatory process has an impact on the carcinogenesis at all stages, namely cancer initiation, promotion and progression. Among all the components of the inflammatory process, a major role as a factor influencing the process of malignant transformation is played by the proinflammatory and anti-inflammatory cytokines. Many studies revealed that a regular intake of NSAIDs significantly reduces the risk of developing CRC. This fact might represent indirect evidence that the inflammatory process promotes neoplasia in CRC. It still remains an open question what role inflammation plays in neoplasia in CRC and whether it plays the same role in both aforementioned pathways of carcinogenesis in CRC. The aim of our study is to determine the effect of inflammatory processes on CRC by determining the level of various cytokines in inflammatory infiltration.

Material and methods

The study involved 4500 patients in whom 694 colon polyps were removed. The patients were between 25 to 82 years old. After detailed verification by two independent pathologists, the studies were done on 144 selected tissue samples. The whole material was fixed in 10% buffered formalin and processed accordi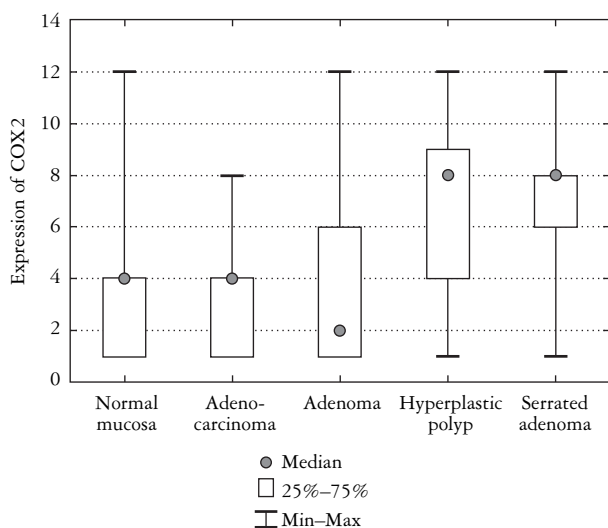ng to a standard protocol. Finally, paraffin blocks were prepared. The inclusion criteria for material used in this study consisted of two main issues, e.g.: the clear-cut diagnosis of mucosal changes that fit the classification criteria and the presence of sufficient material for further work. For further studies, selected samples of large bowel lesions were divided into groups such as adenocarcinoma, adenomas (A), serrated adenomas (SA), hyperplastic polyps (HP) and normal mucosa (as a control group). After preliminary evaluation of hematoxillin and eosin slides, the material was selected for immunohistochemical studies. We used primary antibodies against cyclooxygenase-2 (COX-2), interleukin (IL)-1β, TNF-α, IL-4, IL-10 and for detection – EnVision system (DAKO). Antigen expression evaluation in inflammatory infiltration of selected lesions was carried out using modified Remmele-Stegner scale according to the intensity of expression and the number of positively expressed cells/tissue area (ranging from 1 – lowest expression to 16 – highest expression). The analysis was performed at 20× original objective magnification for each of the studied antibodies on 3 representative and randomly selected areas. The results were analyzed statistically using the nonparametric Kruskal-Wallis test at a fixed level of significance of 0.05.

Results

COX-2 levels in inflammatory infiltration cells

The morphological analysis revealed an expression of COX-2 in inflammatory infiltration cells of all investigated groups (Fig. 1, Table I). Statistical analysis showed a significantly increased level of COX-2 in a group of SA patients compared to normal cells and significantly increased levels of COX-2 in a group of SA patients compared to A patients. No differences were observed between CRC and normal cells. Moreover, no significant difference was observed between A, HP and normal cells.

Cytokine IL-10 levels in inflammatory infiltration cells

The group of A and SA patients exhibited an increased level of IL-10 compared to normal cells but without statistical significance (Fig. 2). Furthermore, no significant correlation was observed regarding IL-10 levels between the patient group of HP, A, SA and normal cells.
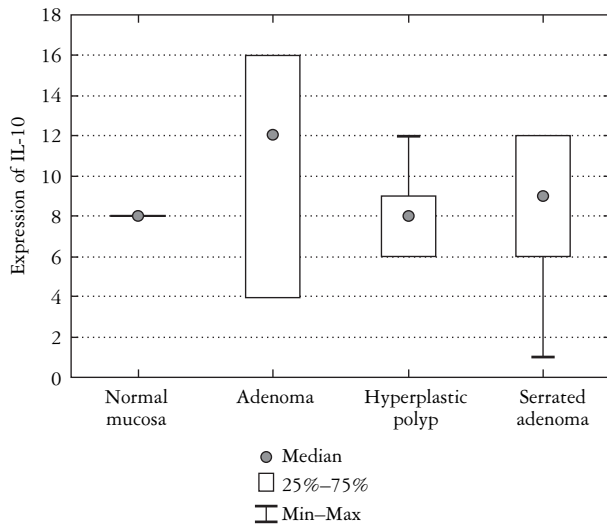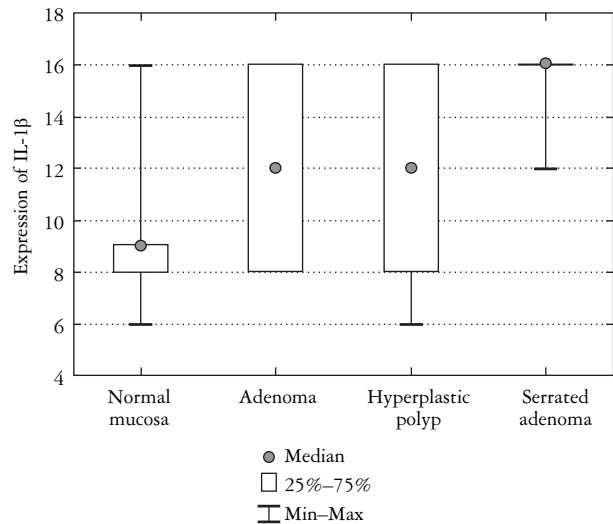
Cytokine IL-1β levels in inflammatory infiltration cells

The level of IL-1β in inflammatory infiltration cells of patients with SA was significantly higher than that of the normal cells (Fig. 3, Table II). We also observed that the group of A patients exhibited an increased level of IL-1β compared to controls, which was of statistical significance. Moreover, in the group of patients

Fig. 1. Average expression of COX-2

Table I. Correlation of cyclooxygenase-2 expression

CYCLOOXYGENASE-2	NORMAL MUCOSA	ADENOCARCINOMA	ADENOMA	HYPERPLASTIC POLYP	SERRATED ADENOMA
Normal mucosa	–	NS	NS	NS	0.000000
Adenocarcinoma	NS	–	NS	NS	0.000000
Adenoma	NS	NS	–	NS	0.000001
Hyperplastic polyp	NS	NS	NS	–	NS
Serrated adenoma	0.000000	0.000000	0.000001	NS	–

NS – not significant

Fig. 2. Average expression of IL-10

Fig. 3. Average expression of IL-1β

with SA, the level of IL-1β was significantly higher than in the group of patients with A. In addition, we observed no difference regarding the IL-1β level between the group of patients with HP and normal cells.

Cytokine IL-4 levels in inflammatory infiltration cells

Statistical analysis demonstrated significantly higher levels of IL-4 in the group of SA patients as compared to the control group (Fig. 4, Table III). We observed higher levels in the group of SA patients as compared to the group of A patients. Moreover, IL-4 levels were significantly higher in the group of HP as compared to the control group. In addition, no dif-

ferences were observed regarding IL-4 levels in respect to severity of cancer and normal cells.

TNF-α levels in inflammatory infiltration cells

The level of TNF-α in inflammatory infiltration cells of patients with SA was significantly higher than that of the controls as seen in Fig. 5 and Table IV. In addition, the mean level of TNF-α was significantly higher in the group of SA patients as compared to the group of A patients. We observed also a significantly increased level in patients with HP in comparison to healthy mucosa. Furthermore, no differences between cancer and normal inflammatory infiltration cells were seen as regards TNF-α levels.

Table II. Correlation of interleukin 1β expression

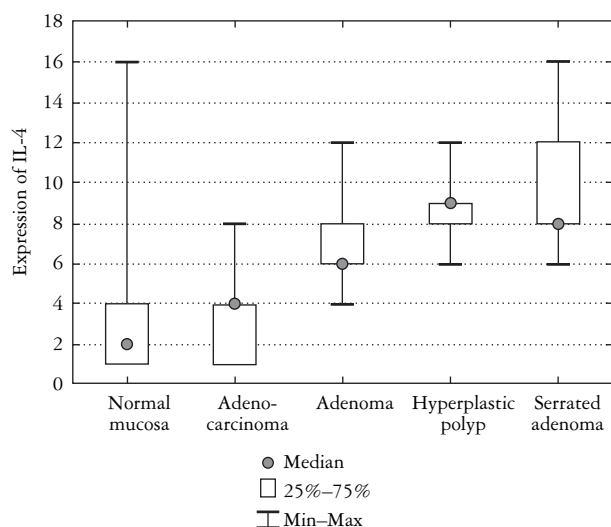| INTERLEUKIN 1β	NORMAL MUCOSA	ADENOMA	HYPERPLASTIC POLYP	SERRATED ADENOMA
Normal mucosa	–	0.035551	NS	0.000000
Adenoma	0.035551	–	NS	0.007446
Hyperplastic polyp	NS	NS	–	0.031317
Serrated adenoma	0.000000	0.007446	0.031317	–

NS – not significant
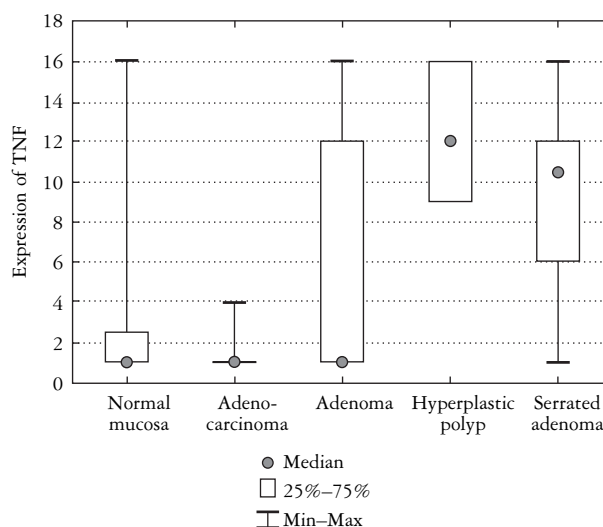
Fig. 4. Average expression of IL-4

Fig. 5. Average expression of TNF-α

Discussion

The inflammatory process has a profound effect on CRC carcinogenesis. In our study we focused on key inflammatory factors such as cytokines IL-10, IL-1β, IL-4, TNF-α and prostaglandin synthase COX-2 in A, SA, HP, adenocarcinomas and non-cancerous mucosa. The results are quite surprising and controversial because they do not share the majority of the existing assumptions in this area. The selected proteins studied in our research are usually described in the publications as a reflection of the intensity of the inflammatory process in neoplasia of colorectal cancer.

The prostaglandin synthase COX-2 is an enzyme synthesizing prostaglandins, in particular PGE-2. The latter, as it was proved by others, affects the progression of CRC. Many scientists in their research showed that in CRC a significant increase in COX-2 levels has been observed [3, 4]. However, Kawasaki *et al.* in their studies regarding the two possible ways of carcinogenesis in CRC showed that the increased expression of COX-2 is not related to an alternative pathway. They found a lower level of expression of COX-2 in serrated adenomas in contrast to traditional adenomas [5]. These results were also confirmed by Angelo *et al.* They found a higher expression of COX-2 in the non-

Table III. Correlation of interleukin 4 expression

INTERLEUKIN 4	NORMAL MUCOSA	ADENOCARCINOMA	ADENOMA	HYPERPLASTIC POLYP	SERRATED ADENOMA
Normal mucosa	–	NS	0.000002	0.000012	0.000000
Adenocarcinoma	NS	–	0.000011	0.000034	0.000000
Adenoma	0.000002	0.000011	–	NS	0.020783
Hyperplastic polyp	0.000012	0.000034	NS	–	NS
Serrated adenoma	0.000000	0.000000	0.020783	NS	–

NS – not significant

Table IV. Correlation of tumor necrosis factor α expression

TUMOR NECROSIS FACTOR α	NORMAL MUCOSA	ADENOCARCINOMA	ADENOMA	HYPERPLASTIC POLYP	SERRATED ADENOMA
Normal mucosa	–	NS	NS	0.000755	0.000001
Adenocarcinoma	NS	–	0.000354	0.000001	0.000000
Adenoma	NS	0.000354	–	0.025119	0.000172
Hyperplastic polyp	0.000755	0.000001	0.025119	–	NS
Serrated adenoma	0.000001	0.000000	0.000172	NS	–

NS – not significant

serrated adenoma comparing to HPP, MP with SSA and adenoma and SSA [3]. But our study does not confirm the results shown in the previously mentioned publication. In our study there was no difference in COX-2 expression between cancer and normal tissue. Our study also showed different results in the expression of COX-2 in the carcinogenesis in CRC. According to our studies, the expression of COX-2 is significantly higher in the so-called serrated pathway. And it was expressed by COX-2 levels in serrated adenoma to be compared to adenocarcinoma. Based on our present research, the results might support the idea of inflammation as a risk factor for CRC. Such findings could explain prevention of colorectal cancer by NSAIDs, till now an unknown mechanism [6]. Patients with familial adenomatous polyposis who regular use NSAIDs reduce the number and size of polyps [7]. This has been confirmed many times by scientific studies. Recently a study by Din *et al.* not only confirmed the effectiveness of NSAIDs in reducing the risk of disease but also indicated that the lowest effective dose should be used. They concluded their research that FAP patients should consume regularly on a daily basis 75 mg aspirin for at least one year in order to effectively reduce the risk of CRC [8]. Anti-inflammatory effects of classical NSAIDs is known as the result of inhibition of COX-2. So, the level of expression of COX-2 is here the most important in the efficacy of preventive NSAIDs activity in CRC. As already mentioned NSAIDs have been proven to reduce the risk of colorectal carcinogenesis. This hypothesis is confirmed by our studies as we have shown elevated levels of COX-2 in SA in particular and to a small extent in the adenoma. Additionally, not increased expression of COX-2 in cancer might suggest the ineffectiveness of NSAIDs in the tumor treatment with the absence of tumor regression. Yet another finding of our study, that is the different levels of expression of COX-2 in cancerogenesis in different pathways might support an explanation of the phenomenon of different preventive activities effectiveness according to NSAIDs use. It could be explained by our finding that the serrated pathway has significantly higher levels of expression of COX-2 compared to the classical way. So the effectiveness of cancer prevention by the use of NSAIDs might have a greater impact in patients whose tumors were formed in an alternative way.

The next inflammatory factor that has been investigated is IL-10. Until now the role of IL-10 in carcinogenesis has been unclear. Some authors suggest that it plays a role as a pro-inflammatory factor, but the others state that it is an anti-inflammatory one. According to our data, the average level of IL-10 in A, SA and HP was slightly higher than in normal tissue, but this is not statistically significant. These small changes probably have no significance in neoplasia of CRC. Cecev *et*

al. showed the differences in the level of IL-10 between the CRC differentiation levels. The highest level of IL-10 was found in well-differentiated tumor, and the lowest in poorly differentiated cases. This would suggest the anti-inflammatory role of IL-10 in CRC [9]. Yet other studies of IL-10 related its levels in cancer patients according to the TNM. In these studies it has been shown that IL-10 is a proinflammatory cytokine because the highest level appeared in the fourth stage, compared to the other three stages of CRC [10, 11]. However the analysis of results of our study confirmed the fact that the level of IL-10 has no effect on the inflammatory process in neoplasia of CRC. According to our data, the level of IL-10 is likely to depend on the individual patient's basis. Based only on the level of expression of IL-10 we are unable to establish any particular pathway relating to the inflammatory process affecting the carcinogenesis of colorectal cancer.

Another important pro-inflammatory factor is IL-1β. Interleukin 1β plays an important role in the regulation of COX-2. There is a significant relationship between the concentration of COX-2 and IL-1β. Such statement could be confirmed by our findings. Malhofner *et al.* [12] have demonstrated an increased expression of IL-1β in the epithelium of CRC. However, on the basis of our study, an increased expression of IL-1β in SA and A as compared to normal tissue could be confirmed. And it is worth highlighting that IL-1β is another pro-inflammatory factor that has an increased expression in SA. To our knowledge it is the first report on this issue as the expression of the IL-1 interleukin has not yet been determined in SA and A earlier. Interleukin 1β also has been proven as having influence on the growth CRC. According to studies by others, IL-1β probably takes part in the development of metastases. This phenomenon could be explained by involvement of IL-1 in the stimulation metalloproteinases which are responsible for tissue degradation needed for metastasis [13]. As one can see IL-1β is an important factor both on the carcinogenesis as well as progression and metastasis in CRC. As it was described above, IL-1β might have an indirect influence on tumor progression. This factor should deserve attention and there is a need for further research on its role.

Interleukin 4 is produced by mast cells, basophils and activated T lymphocytes [14]. A study conducted by Galon *et al.* shows a significantly elevated level of IL-4 in patients with CRC as compared to healthy ones [15]. But results of our studies do not confirm this statement. According to our studies, no difference regarding the IL-4 level was observed between cancer cells and normal cells. The expression level of IL-4 was elevated in A, SA and HP as compared to normal tissue. A higher level of pro-inflammatory factor in this case was in SA in comparison to A. This is the first report on such phenomenon. Kanei reported that IL-4 inhibited colon cancer cell-cell adhesion by decreasing

the expression of E-cadherin and CEA molecule. However, the authors of those studies were unable to show that this process has an effect on metastasis and invasion. Moreover, just cited studies did not show any connection between the increased level of IL-4 and cell proliferation [16]. Our studies partly confirmed previously published data. No differences between levels of IL-4 in cancer and normal cells may lead to the theory that IL-4 has no effect on metastasis and invasion at all. But based on results of our studies, the increased level of IL-4 in A, SA and HP means that the cytokine has an influence on CRC neoplasia. However, it still remains an unanswered question what role IL-4 plays in neoplasia of CRC. Whether it plays the role such as another pro-inflammatory cytokine or maybe as an anti-inflammatory one.

Tumor necrosis factor α is a pro-inflammatory cytokine. Tumor necrosis factor α is primarily released by activated monocytes and macrophages. This cytokine also stimulates the liver to produce acute phase proteins including CRP. Balkwill *et al.* have shown that if the level of TNF-α is elevated in patients with CRC they have poor prognosis [17]. However, our study did not show any increase in expression of TNF-α in cancer cells. Others exploring the role of inflammation in oncology [18] especially model with treatment by use anti-TNF-α in mice ablates influence on the expression of COX-2 by normalization of its level and decreased the level of oncogenes c-myc [19]. Additionally, Poutahidis *et al.* have shown that anti-TNF-α treatment with in mice ablates cancer decreased the levels of K-ras genes [20]. Our studies could confirm the previously published results as there was found a significantly higher expression of TNF-α in SA and HP compared to normal tissue. And also a higher average level of TNF-α expression in adenomas compared to the average expression level in the normal tissue. Analysis of our results might support the impression that TNF-α have an influence on the CRC neoplasia.

In conclusion, our study confirms the hypothesis that inflammation has a major effect in carcinogenesis of colorectal cancer. Additionally, this study also showed that the inflammatory process has no influence on the final form of cancer. Interestingly, the cancer has not increased expression of inflammatory factors. Our studies also showed a difference in carcinogenesis of CRC. Studies showed a greater effect of inflammatory process in carcinogenesis of CRC in the serrated way as compared to the classic way. In the serrated way, there was a higher expression of all the inflammatory factors. Our results significantly differ from previously published ones about the inflammatory process in the carcinogenesis of CRC, as it was shown above. But based on our study one can explain a previously unknown mechanism of action of NSAIDs as a preventive agent reducing risk of CRC incidence and prevention of cancer in familial polyposis patients.

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