

Myeloperoxidase-Positive Cell Infiltration in Colorectal Carcinogenesis as Indicator of Colorectal Cancer Risk

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Abstract

Colorectal mucosa is targeted by toxic agents, which can initiate or promote colon cancer. The mechanism of damage might be a focal irritation with loss of normal epithelial cell barrier function. Genetic alterations in tumors may also affect host inflammatory response. The aim of this study was to define the extent of inflammation in colorectal mucosa, along colorectal carcinogenesis, and in microsatellite stable and unstable colorectal carcinomas. We collected 103 samples of normal colorectal mucosa from 65 patients (35 with colorectal cancer or adenoma, 8 with inflammatory bowel diseases, and 22 controls with normal colonoscopy). We also examined 24 aberrant crypt foci, 14 hyperplastic polyps, 16 adenomas, and 67 samples of colorectal carcinoma. Immunohistochemistry was used to count myeloperoxidase (MPO)-positive cells (neutrophils and monocytes) in $\times 100$

optical fields under a light microscope. Patients with colorectal tumors had a higher mean number of MPO-positive cells in normal mucosa than controls (mean \pm SD, 2.7 ± 2.0 versus 1.4 ± 1.4 ; $P = 0.017$). MPO-positive cell number was tightly linked to dysplasia in aberrant crypt foci and adenomas, and it was higher in carcinomas microsatellite unstable than those microsatellite stable (21.6 ± 15.5 versus 11.9 ± 8.0 ; $P < 0.01$). MPO immunohistochemistry is a simple and reliable technique for the quantification of inflammation in colorectal mucosa, and it may be a potential marker of colorectal cancer risk. Microsatellite instability seems to influence host immune responses to colorectal carcinoma. These observations strongly support a key role of inflammation in colorectal carcinogenesis. (Cancer Epidemiol Biomarkers Prev 2008;17(9):2291–7)

Background

The relationship between chronic inflammation and risk of cancer is strongly supported by several observations on cancer of many tissues and organs. About 15% of all cancers have been attributed to infectious agents (1), and chronic inflammation caused by chemical and physical agents has been associated with risk of malignancy (2). Colorectal mucosa is targeted by several toxic agents present in the diet or produced by colonic bacteria, which can initiate or promote colon cancer. Examples of such compounds are pyrolysis products in cooked food (3) and products of fat digestion. Moreover, Western diets cause increased body fat and obesity, which may be linked to colonic inflammation, and to increased colorectal cancer risk (4). The mechanism of damage might be

a focal irritation in the mucosa with loss of normal epithelial cell barrier function (5). Experimental and human studies have shown that several compounds (anti-inflammatory drugs and antioxidants) can inhibit colon carcinogenesis (6). In the bowel, inflammation of the mucosa is the prominent feature of Crohn's disease and ulcerative colitis (called inflammatory bowel diseases, IBD) both considered at increased risk for colorectal cancer (7, 8). In IBD, cancer risk is associated with site, extent, and duration of inflammation (9). Focal mucosal inflammation can increase cell proliferation and mutations and can be responsible for the neoplastic transformation of the intestinal epithelium, giving rise to colorectal focal early precursor lesions as aberrant crypt foci (ACF). ACF may be considered the earliest event in colorectal carcinogenesis both in experimental models (10) and in humans (11, 12). In particular, dysplastic ACF (microadenomas) seem to have a high potential to progress toward cancer development, although also nondysplastic ACF may have a role in colorectal carcinogenesis (13). Furthermore, some evidence suggests that also genetic alterations, and in particular, genetic instability at microsatellites, may be linked to increased inflammatory infiltrate in tumors at different sites, including colorectal (14, 15).

The mechanisms of malignant transformation driven by inflammation involve production of cytokines, inhibition of apoptosis, and increased cell proliferation.

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Colonic inflammation is pathologically defined as the presence in the colonic mucosa of cells involved in the immunologic defense system. In particular, accumulation and infiltration by neutrophils and monocytes is a prominent feature in the local inflammatory process in IBD. Thus, a specific neutrophil and monocyte marker can be useful to evaluate the presence and extent of colonic inflammation in normal mucosa and in colorectal premalignant and neoplastic lesions. Among such possible markers, we chose myeloperoxidase (MPO), an enzyme which is contained in lysosomes of neutrophils and, to a much lesser extent, of monocytes and tissue macrophages (16). Thus, the principal aim of the present study was to evaluate the presence and to define the extent of inflammation in colorectal mucosa and along colorectal carcinogenesis, counting MPO-positive cells in histologic sections of normal colorectal mucosa (NM), as well as early colorectal lesions, i.e., ACF, and to compare these counts with those obtained in later stages, i.e., in polyps and cancer, using a simple immunohistochemical technique. Differences in MPO-positive cell infiltration were also searched for between microsatellite unstable (MSI) and stable (MSS) colorectal carcinomas.

Materials and Methods

Study Subjects and Materials. We collected 103 samples of NM from 65 patients (26 women and 39 men; mean age, 61 y), during colonoscopy, or after operation on surgical specimens, and fixed in a 10% formalin solution (Table 1). Of these, 35 had colonic or rectal carcinoma (15 patients, 2 of them with familial adenomatous polyposis), or one or more colorectal adenomas (20 patients; NM/K). Eight patients were on follow-up colonoscopies for IBD (ulcerative colitis, six patients; Crohn's disease, two patients; NM/IBD). For these patients, biopsies were taken on colorectal mucosa with no macroscopic sign of inflammation. Twenty-two subjects had normal colonoscopy and were used as controls (NM/N). Twenty-four ACF were collected, as previously described (12, 17), from colorectal surgical specimens after operation for colorectal cancer from nine patients (two of them had familial adenomatous polyposis). Twenty ACF of 24 showed dysplasia at histology, and only 4 were nondysplastic. Moreover, we examined formalin-fixed paraffin-embedded histologic sections of 14 hyperplastic polyps (HP) from 14 patients, 16 adenomas (Ad) from 13 patients, and 67 samples of colorectal carcinoma (K) from 65 patients. Among the 67 samples of carcinoma, the presence of instability at DNA

microsatellites could be determined in 55 samples (27 MSI and 28 MSS).

All patients were asked to give an informed written consent to the study protocol, which was approved by the local Ethical Committee (University Hospital of Modena).

Immunohistochemistry. Samples were fixed in a 10% formalin solution for 1 h. They were then dehydrated, embedded in paraffin wax, and 3- μ m sections were placed on SuperFrost Plus microscope slides (Menzel). Before immunohistochemistry, routine histology of all tissue samples was carried out after H&E staining of the sections. Slides were dried overnight at 37°C, dewaxed in two changes of fresh xylene, and rehydrated in a descending alcohol series. Antigen retrieval involved treatment with a protease (Pronase 1:20; DakoCytomation) for 7 min at 37°C. Before immunohistochemical staining, the sections were washed with PBS and blocked with 20% Swine Serum (Normal; DakoCytomation) in PBS for 30 min to reduce nonspecific antibody binding. Primary antibody was then applied at appropriate dilutions for 30 min at room temperature. Polyclonal rabbit anti-human MPO antibody (DakoCytomation) was used as the primary antibody at a dilution of 1:200 in PBS. After washing with PBS, monoclonal antibodies against rabbit immunoglobulin were applied as link antibodies (1:30; Dako) for 30 min. Polyclonal Rabbit Anti-mouse Immunoglobulins/AP (1:20; DakoCytomation) secondary antibody was incubated for 30 min, and further detection was carried out using APAAP mouse monoclonal antibodies (1:35; DakoCytomation) in accordance with the manufacturer's instructions. Color was developed with a solution of New-Fuchsin and Naphtol AS-BI Phosphate (Sigma-Aldrich). Slides were counterstained with Mayer's hematoxylin, dehydrated in an ascending alcohol series, and covered with a coverslip (histo-vitrex mounting medium; Carlo Erba). For all these procedures, staining without the primary antibody was done as a negative control.

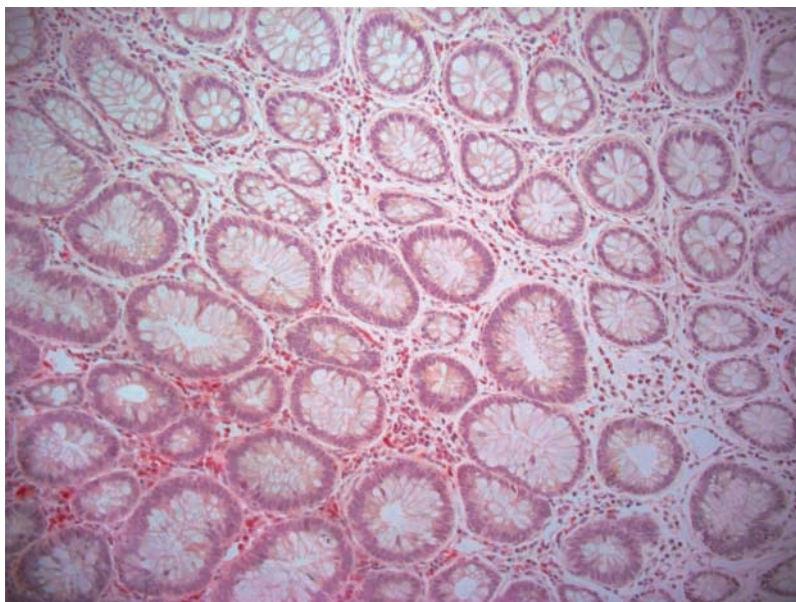
Scoring System. One slide for each sample was scored in most cases. A quantitative score was used, counting the number of stained cells per $\times 100$ optical fields under a light microscope. We excluded positive cells located into capillary vessels around or into the lesions (adenoma and carcinoma). In carcinoma, we chose fields just around the tumor, avoiding microvessels, necrosis, and lysis, and we focused on areas with the highest density of staining. For each slide, a mean staining score was calculated. Then, overall mean staining scores were obtained for each lesion. For normal mucosa, when more

Table 1. Number of patients, number of samples, and overall mean number (\pm SD) of $\times 100$ optical fields counted for MPO-positive cells in colorectal mucosa and in colorectal lesions

Colorectal tissue	No. of patients	No. of samples	Mean no. of optical fields (\pm SD)
Normal mucosa	65	103	47 (± 28)*
ACF	9	24	31 (± 9)
Hyperplastic polyp	14	14	27 (± 7)
Adenoma	13	16	32 (± 13)
Carcinoma	65	67	43 (± 13)
Total	140	189	

*For NM, the mean was calculated for patients and not for samples.

Figure 1. Normal colorectal mucosa (*top right*) and dysplastic crypts in an ACF (*bottom left*). Note the higher stromal infiltration of MPO-positive (*red-stained*) cells among aberrant crypts than among normal ones. Magnification, $\times 20$.



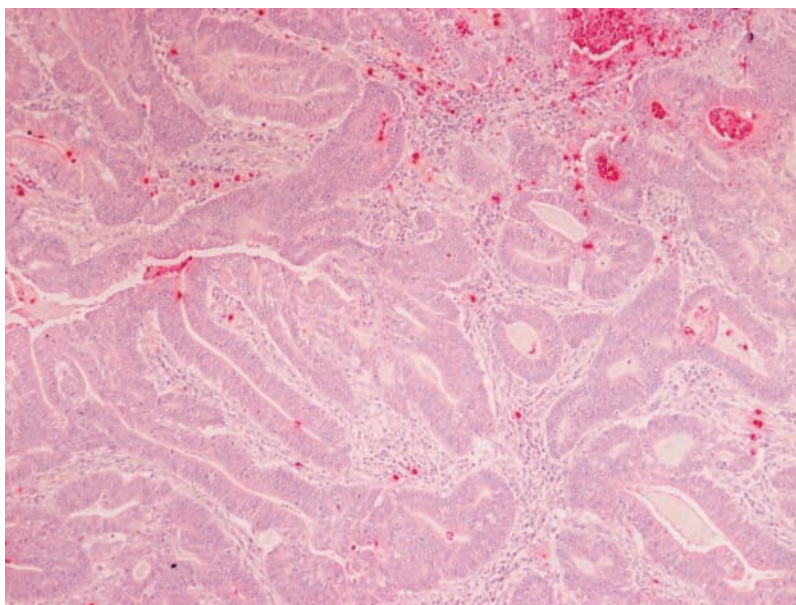
than one sample for each patient was available, we calculated the mean number of MPO-positive cells for each patient, and then we entered this number for the calculation of the overall mean of the whole group. The scores were compared across groups and, for normal mucosa, according to the disease of the patient, and for carcinomas, according to DNA microsatellite status. Slides of samples of NM and carcinomas were coded and scored blindly. Moreover, five slides for each group of normal mucosa and lesions were scored by two independent observers, and the results were compared to test reproducibility of scoring. When the staining scores were discordant, and this happened only in a few cases,

an agreement was reached by the two observers at a light microscope with double view.

Microsatellite Instability. DNA microsatellite instability in carcinomas was evaluated using two mononucleotide markers (BAT25 and BAT26), as previously described in detail (18).

Statistical Data Analysis. Cohen's κ coefficient (19) was used to estimate the level of agreement between two independent observers on single-optic-field scores of MPO-positive cells for five slides for each group of lesions. ANOVA (with Levene, Welch, and Brown-Forsythe tests; ref. 20) was used to evaluate the differ-

Figure 2. Colorectal carcinoma at magnification $\times 10$. Note that the density of MPO-positive cells is higher in the upper part of the figure. Moreover, in the top right corner, necrotic and not viable cells are evident, which, as explained in the Materials and Methods, were not considered in the MPO score.



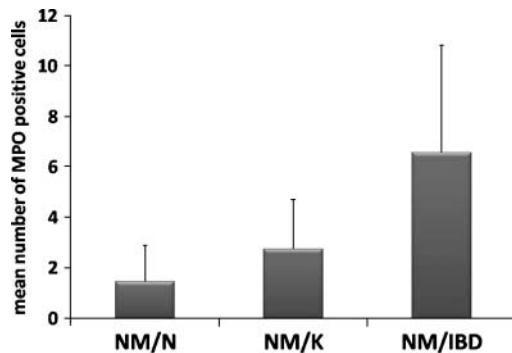


Figure 3. Overall mean number of MPO-positive cells per $\times 100$ optical fields in the mucosa of subjects with normal colonoscopy (NM/N), in the normal mucosa of patients with colorectal tumors (NM/K), and with chronic IBDs (NM/IBD). The overall difference among groups is significant ($P < 0.01$), as it is between NM/N and NM/K ($P = 0.017$), and between NM/N and NM/IBD ($P = 0.032$), whereas no significant difference is evident between NM/K and NM/IBD.

ences in mean MPO staining scores among the three groups of NM, and among NM, hyperplastic polyps, ACF, adenomas and carcinomas groups. Then, differences of mean MPO scores between groups were compared using appropriate *post hoc* tests (i.e., Tamhane, Dunnnett T3, and C, Games-Howell tests). Moreover, Cuzick test (21) was used to evaluate the trend of increasing MPO staining among groups. The difference in mean MPO staining scores between dysplastic and nondysplastic ACF was compared with the Wilcoxon Mann-Whitney test (taking into account the small sample size of the latter group), and that between MSI and MSS carcinomas with the Student's *t* test. Differences were considered significant when the probability of chance was $< 5\%$.

Results

The quality of staining was usually very good. MPO immunostaining was evident as red spots in the cytoplasm of neutrophils and monocytes, allowing their easy identification even at low magnification. Examples of MPO staining are shown in Fig. 1 (NM and aberrant crypt focus) and 2 (colorectal carcinoma). For each group of colorectal tissue and lesion, a mean of at least 25×100 optical fields was scored (Table 1). The number of optical fields examined per group was higher in normal mucosa because more than one biopsy was often examined per single patient, although biopsies taken during endoscopy were usually small.

The overall agreement between the two observers on the staining scores was good, and only in a few cases of carcinoma slides were reevaluated by the observers or by a third one. For the samples examined independently by two observers, the Cohen's κ was 0.73, indicating full agreement.

In each sample of NM, some MPO-positive cells were always found. The overall difference of the mean number of MPO-positive cells among the groups of NM

examined was highly significant ($P < 0.01$; Fig. 3). The number was highest in the mucosa of patients with IBD (mean \pm SD, 6.6 ± 4.2) and significantly higher in the mucosa of patients with adenoma or cancer than in subjects with normal colon (mean \pm SD, 2.7 ± 2.0 versus 1.4 ± 1.4 ; $P = 0.017$). Figure 4 shows the overall mean number of MPO-positive cells in all groups examined. The overall difference among groups was highly significant, as it was the trend of increasing staining from NM through carcinomas (by Cuzick test for trend). All pairs of mean staining scores in the groups examined were statistically different, except NM-HP and ACF-Ad pairs. With respect to NM (2.8 ± 2.7), MPO-positive cells infiltration was progressively higher in the samples of ACF (5.6 ± 2.5 ; $P < 0.01$ versus NM), adenoma (7.8 ± 4.3), and carcinoma (16.2 ± 12.3), whereas in hyperplastic polyps, it was similar to that of normal mucosa (2.9 ± 2.3). In ACF, the overall mean number of MPO-positive cells was higher in dysplastic ACF than in nondysplastic ACF (6.2 ± 2.2 versus 2.7 ± 1.2 , respectively; $P = 0.01$; Fig. 5). Interestingly, the amount of MPO-positive cell infiltration was related to the degree of dysplasia in the lesion. In carcinoma, positive cells were not evenly distributed in the tissue, but tended to gather in some areas, usually at the edge of the neoplastic tissue, where they were counted (Fig. 2), and sometimes also in the stroma inside the tumor, and even among the neoplastic cells (intraepithelial-positive cells). When carcinomas were considered, according to DNA microsatellite status, the mean number of MPO-positive cells was higher in carcinomas with microsatellite instability than in those MSS (21.6 ± 15.5 versus 11.9 ± 8.0 ; $P < 0.01$; Fig. 6).

Discussion

The present study was undertaken to quantify the presence of inflammation along colorectal carcinogenesis. We have chosen MPO as a specific marker of neutrophil and monocyte stromal infiltration in normal mucosa and in colorectal premalignant lesions and carcinoma. This is one of the main enzymes released upon neutrophil activation, and it is a heme protein that

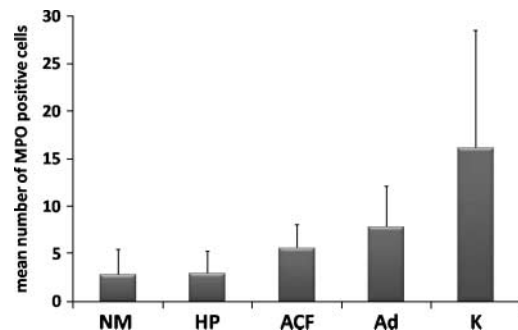


Figure 4. Overall mean number of MPO-positive cells per $\times 100$ optical fields in normal colorectal mucosa (NM), hyperplastic polyps (HP), aberrant crypt foci (ACF), adenomas (Ad), and carcinomas (K). Overall difference among groups is highly significant ($P < 0.01$), as it is the trend of increasing MPO staining from NM through carcinomas ($P < 0.01$). All differences in mean MPO staining between groups are significant, except for NM-HP and ACF-Ad pairs.

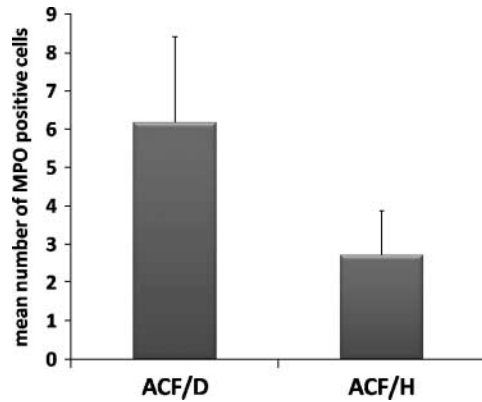


Figure 5. Overall mean number of MPO-positive cells per $\times 100$ optical fields in dyplastic (ACF/D, 20 samples) and nondyplastic (ACF/H, 4 samples) ACF. The difference between groups is significant ($P = 0.01$, Wilcoxon-Mann-Whitney test).

generates cytotoxic oxidants. MPO has long been considered a key constituent of the neutrophil cytotoxic armament by catalyzing the formation of hypochlorous acid, a potent oxidant with bactericidal activity *in vitro* (22). MPO staining is clear and easy to score and is simple to detect and able to quantify the inflammation. It has been reported that estimating the amount of tumor-infiltrating immune cells on H&E-stained sections is sensitive to interobserver variability (23) and underestimates the true number of leukocytes in a tumor. In the present study, a good interobserver agreement for the count of MPO-positive cells on the same optical fields was found, confirming the reproducibility of the method. MPO immunostaining was more difficult to define in cancer tissue due to necrosis and intralesion variations. We chose, such as in other studies, to avoid counting areas with necrosis and focusing on the most labeled ones (24, 25).

The most important findings of the present study are the following:

1. Inflammation, as measured by neutrophil and monocyte infiltration, is present at low level even in normal colorectal mucosa of subject with no lesion at endoscopy. As expected, MPO-positive cell infiltration was higher in the mucosa of patients with IBD.
2. Patients with colorectal tumors had a significant higher number of MPO-positive cells in normal mucosa than controls.
3. MPO-positive cell number increases during colorectal carcinogenesis from normal mucosa to colorectal cancer. In particular, a new finding is the tight correlation between number of MPO-positive cells and dysplasia in ACF, considered the earliest event in colon carcinogenesis (26, 27). Indeed, the mean number of MPO-positive cells was not different in ACF and adenomas. On the other hand, hyperplastic polyps had low levels of infiltration, similar to normal mucosa. Adenomas, and to a greater extent, carcinomas, showed higher levels of infiltration,

although in these later lesions, the phenomenon may be caused by the neoplastic transformation itself, and thus, it should be considered a secondary event.

4. Carcinomas had the highest MPO-positive cell infiltration, and this was more evident in MSI than in MSS colorectal carcinoma. Indeed, defects in mismatch repair genes, which are the hallmark of Hereditary NonPolyposis Colorectal Cancer syndrome, result in the introduction of mutations at repetitive nucleotide sequences in the genome (microsatellites) that are potentially immunogenic. This study shows that genetic alterations may influence per se neutrophil infiltration. This is in line with other works that support the role of inflammation and immunologic response in tumors with MSI (14, 15). It is widely accepted that the putative carcinogenic activity of neutrophils is, at least in part, due to the generation of DNA damage and of mutagenic reactive oxygen species (5). Furthermore, it has been established that the type of DNA base modifications, as detected in target cells exposed to H_2O_2 , is similar to the damage induced by activated neutrophils. Also, hypochlorous acid has been shown to be an inhibitor of DNA strand break repair (28). Hypochlorous acid is a relatively stable and membrane-diffusible molecule that can reach the nucleus. It has the potential to interact with many different cellular proteins, including DNA repair proteins (29). Interestingly, MSI may be caused by increased epithelial cell proliferation associated with inflammation also in non-neoplastic colonic tissue (30). Inflamed colonic mucosa shows alterations in these molecular pathways even before any histologic evidence of dysplasia or cancer (31). In experimental models, it has been shown that the number of gene mutations in individually growing tumors was associated with the number of infiltrating neutrophils (32, 33). Our results are in line with these observations, linking inflammation and MSI (34).

The link between inflammation and colorectal cancer development is supported by several experimental, epidemiologic, and clinical observational studies, both

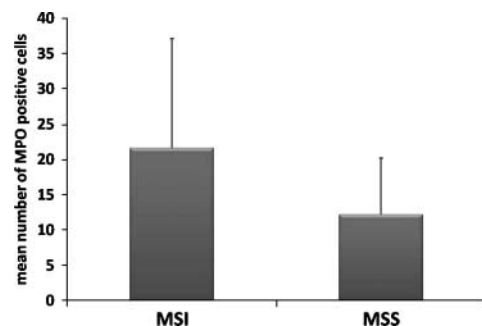


Figure 6. Overall mean number of MPO-positive cells per $\times 100$ optical fields in carcinomas with (MSI; $n = 27$) and without (MSS; $n = 28$) microsatellite instability. The difference between groups is significant ($P < 0.01$, Student's *t* test).

in ulcerative colitis (31, 35), and colorectal cancer not related to ulcerative colitis (36-38) possibly caused by bacteria, through gene alterations (39, 40). An interesting link between some markers of insulin resistance and colonic inflammation has also been hypothesized (5), and even fatigue and depression seem to influence colonic inflammation in irritable bowel syndrome (41). Moreover, obese people have higher levels of fecal calprotectin, another specific marker of neutrophil and monocyte infiltration, suggesting that the adipose tissue may modulate inflammation and immune functions also in the large bowel (4).

Compromised intestinal epithelial barrier function in IBD has been well-documented leading to hyper-permeability to luminal macromolecular products. An increased intestinal epithelial permeability may cause or exaggerate inflammation in the intestine (5, 42). It is not clear whether the barrier defect is a primary event or it is a consequence of the inflammation, although most published data show a correlation between neutrophils and transmembrane proteins of the tight junction complex (43). This work brings further support to the key role of inflammatory processes, and in particular, of neutrophils, to the initial phases of this progression.

In conclusion, the results of the present study suggest that the colonic mucosa is in a state of chronic inflammation, more evident in patients with colorectal neoplasia or IBDs. MPO immunohistochemistry is a simple and reproducible method to detect inflammation in colorectal mucosa. Genetic alterations and, in particular, instability at DNA microsatellites could influence inflammatory and host immune responses to colorectal carcinoma. These observations strongly support a key role of inflammation in particular in the early steps of colorectal cancer development. Thus, it should be tested the feasibility to use inflammation-related markers, such as MPO, as possible indicators of colorectal cancer risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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