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Peritumoral inflammatory reaction in colon cancer. Histological and immunohistochemical study

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Abstract

Colorectal cancer is one of the most frequent malignancies with an increasing incidence and prevalence. As in other malignancies, nor etiology, neither pathogenesis of colorectal cancer are well known. The link between inflammation and colorectal cancer has become a major concern in the past 20 years, since several clinical trials have shown that patients with chronic inflammatory intestinal diseases have a much higher risk of colorectal neoplasm development. In our study, we analyzed peritumoral inflammatory reaction from histological and immunohistochemical point of view, in 23 cases of stage III colon adenocarcinoma, operated during 2014. The immunohistochemical techniques were used in order to emphasize B-lymphocytes, T-lymphocytes, macrophages, mast cells and blood vessels. In all cases, we have noted the involvement of inflammatory cells present in peritumoral and tumoral stroma, in variable degrees, regardless the differentiation of the neoplasm or other known histological feature. In particular, the macrophages were the most numerous, especially in areas of tumoral necrosis, but also present in the lumen of tumoral glands, or even within tumoral cell islands. Mast cells appeared more abundant in the tumor stroma around blood vessels and were absent in the areas of tumor necrosis, while B-cells were almost absent. Tumor stroma showed a well-developed vascular network, consisting mainly of small vessels that do not seem to correlate with the intensity of the inflammatory reaction.

Keywords: colon cancer, inflammatory cells, chronic inflammation, mast cells, macrophages.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide [1]. According to some authors [2], CRC is the fourth in men and third in women as frequency, with a mortality that ranks second after lung cancer [3], resulting in over 800 000 related deaths each year. Worldwide reports show slightly rising incidence both in men and women.

Relatively recent studies counted around 102 900 cases of colon cancer and 39 670 cases of rectal cancer newly diagnosed only in the US in 2010; approximately 51 370 patients died from CRC in the same year, representing approximately 9% of all cancer-related deaths [4].

As in other malignancies, nor etiology, neither pathogenesis of colorectal cancer are not very clear. A number of factors were considered important in causal relationship, such as adenomatous polyps, hereditary syndromes (familial adenomatous polyposis, hereditary non-polyposis colorectal cancer – Lynch syndrome), inflammatory bowel disease, diet, smoking, lifestyle, etc.

The link between inflammation and cancer is a relatively recent concern, although since 1863 Virchow showed that different cancers were detected in areas of chronic inflammation [5]. Furthermore, many clinical and epidemiological studies demonstrated that chronic inflammatory intestinal diseases, namely ulcerative colitis and Crohn's disease have an increased risk of malignant degeneration [6–10]. Recent evidence suggests that between inflammation and cancer there is a close relationship, the malignant cells being capable of inducing a local or systemic inflammation, the activation of transcription factors and major inflammatory cytokine production [11]. In turn, inflammation associated with cancer may influence cell proliferation and survival, angiogenesis, tumor cell migration, invasion and tumor metastasis [11].

In this study, we have proposed to identify inflammatory cells present in the peritumoral stroma and quantify their participation in peritumoral inflammatory reaction.

Materials and Methods

The study was conducted on a total of 23 stage III (TNM) colorectal adenocarcinomas, operated during 2014 in the IInd General Surgery Clinic of Emergency County Hospital of Craiova, Romania. Immediately after surgery, tumor fragments of about 2/1.5 cm were retrieved from

the pathological specimen and then fixed in 10% formalin solution at room temperature for 72 hours. Biological samples with a thickness of 1.5 mm were embedded in histological paraffin according to the protocol used by the laboratory of pathology. Sectioning of biological material was performed with a Microm HM350 rotary microtome, equipped with rotary system of section transfer on water bath. For histological study, we used the classic Hematoxylin–Eosin (HE) and trichromic Goldner–Szekely (GS) stains.

For the immunohistochemical study, 4-μm thickness sections were performed that were collected on poly-L-Lysine covered plates.

For the immunohistochemical study was performed 4-μm thick sections were collected onto slides coated with poly-L-Lysine. Following dewaxing and hydration of histological sections, they were incubated for 30 minutes in a solution of 3% hydrogen peroxide and washed in tap water for five minutes. For the antigen unmasking, the slides were boiled in a microwave oven, in a solution of sodium citrate, pH 6, for 21 minutes (seven cycles of three minutes). After boiling, they were left to cool down for 15 minutes, followed by their wash-up in a buffering solution of phosphate-buffered saline (PBS) and non-specific sites blocking using 2% fat-free milk for 30 minutes. The sections were then incubated with primary antibodies, for 18 hours (overnight), in a refrigerator at 4^0C. In the next day, biotinylated secondary antibody was applied (αMs/αRb) for 30 minutes followed by Streptavidin-HRP passage-30 minutes. The signal was detected with 3,3'-diaminobenzidine (DAB) (Dako) followed by Hematoxylin contrasting, dehydration in alcohol, clarifying in xylene and blades mounting in DPX environment (Fluka).

In our study, we used the following markers (Table 1):

Table 1 – ***Antibodies used in the immunohistochemical study***

Antibody	Code	Clone	Antigen retrieval	Specificity	Dilution	Source
CD20	M0755	L26	Sodium citrate buffer, pH 6	B-lymphocytes	1:100	Dako
CD3	A0452	F7.2.38	Sodium citrate buffer, pH 6	T-lymphocytes	1:100	Dako
CD68	M0814	KP1	Sodium citrate buffer, pH 6	Macrophages	1:200	Dako
Tryptase	M7052	AA1	Sodium citrate buffer, pH 6	Mast cells	1:1000	Abcam
CD34 class II	M7165	QBEnd-10	Sodium citrate buffer, pH 6	Vascular endothelium	1:50	Dako

Results

The histopathological study revealed that all 23 cases were adenocarcinomas, eight of them being well differentiated, 13 moderately differentiated while two were poorly differentiated. The intensity of the inflammatory response was extremely varied from one case to another, regardless the degree of tumor differentiation (Figures 1 and 2). As shown in some well-differentiated cases, the inflammatory reaction was weak, while moderate or intense in others. Generally, we have noticed that the intensity of the inflammatory reaction reached maximum development in the areas of tumor necrosis (Figures 3 and 4). Observed on powerful microscope lens the inflammatory infiltrate from peritumoral and tumoral stroma was mainly formed by lymphocytes, plasma cells and macrophages. In the areas with intense inflammatory infiltrate, we also found more numerous blood vessels, especially congested capillaries. Further, we used immunohistochemistry techniques for selective highlighting of inflammatory cells types. T-lymphocytes were revealed using anti-CD3 antibody, CD3 being a T-cells complex membrane protein with very high specificity, thus largely used for selective differenttiation of lymphocytes populations. In our study, the density of T-lymphocytes varied proportionally with the abundance of inflammatory infiltrate (Figures 5 and 6). In areas of tumor necrosis, we noticed a moderate amount of T-lymphocytes which occurred with a relatively evenly distribution (Figure 7). B-cells were identified using anti CD20 antibody that links a glycosylated phosphoprotein present on B-cells surface. Regardless of the intensity of the inflammatory infiltrate, B-lymphocytes were rarely identified in the peritumoral stroma, showing a low involvement in local inflammatory response (Figure 8).

Anti-CD68 antibody was used to reveal the macrophages. CD68 is a glycoprotein that is found in the cytoplasmic organelles (in large amount in lysosomes) of the monocytic system cells (monocytes, macrophages, Kupffer cells, multinucleated giant cells, osteoclasts). Our study revealed that, from all inflammatory cells populations of the innate immune system, the most abundant were macrophages. The disposal of macrophages in the tumoral stroma of colorectal adenocarcinomas was heterogeneous, with an uneven layout as in the case of T-lymphocytes. Thus, we discovered heavily infiltrated areas with macrophages coexisting with moderate stromal infiltrate areas without an apparent cause that could be histologically identified (Figure 9). The microscopic study allowed us to notice the presence of macrophages not only in the tumoral and peritumoral stroma, but also around cancer cells or into the lumen of tumoral gland (Figure 10). The highest density of macrophages was recorded in tumor necrosis zones (Figure 11), most probably induced by the presence of tumoral tissue debris.

Mast cells were revealed using anti-tryptase, a widely used immunohistochemical method in all research and pathology laboratories, since tryptase is the most abundant biochemical element present in the secretory granules of mast cell. The dissemination of mast cells in the tumoral and peritumoral stroma was completely heterogeneous, in the sense that we have identified areas with low cellularity (Figure 12) and areas with abundant cellularity (Figure 13). The highest density of mast cells was identified around blood vessels (Figure 14), while in tumor necrosis zones mast cells were almost absent (Figure 15).

Given the fact that all neoplasms have a rich blood supply, in this study, we also aimed to highlight the vessels of the tumoral stroma and to correlate their density with the presence of inflammatory cells. For the blood vessels' immunostaining, we have used anti-CD34 antibody directed towards a surface glycoprotein of endothelial cells that acts as an intercellular adhesion factor. As emphasized in our images, in all adenocarcinomas of the colon we found a very well developed vascular network, consisting mainly of capillary vessels,

arterioles and small veins, extremely varied in size (Figure 16). Generally, the majority of blood vessels were disposed around islands of tumoral cells in the absence of inflammatory infiltrate to support their development (Figure 17). This leads us to believe that tumor cells have the ability to induce the formation of new vessels to allow multiplication, growth and metastasis using a pathway that excludes inflammation, the inflammatory response appearing later and becoming maximal in the presence of tumor necrosis.

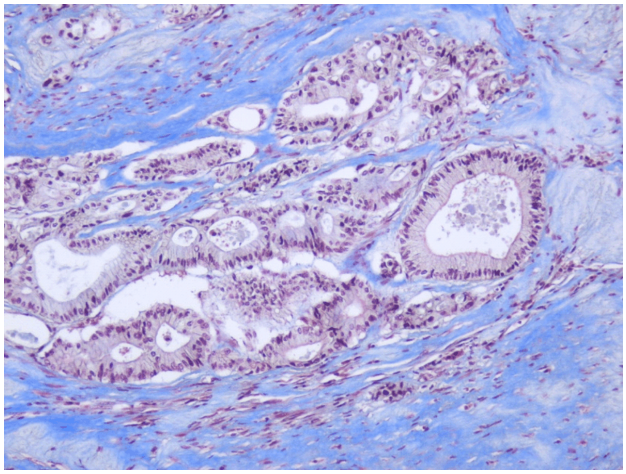

Figure 1 – ***Well-differentiated colon adenocarcinoma with low inflammatory reaction. Trichromic GS staining, ×200.***

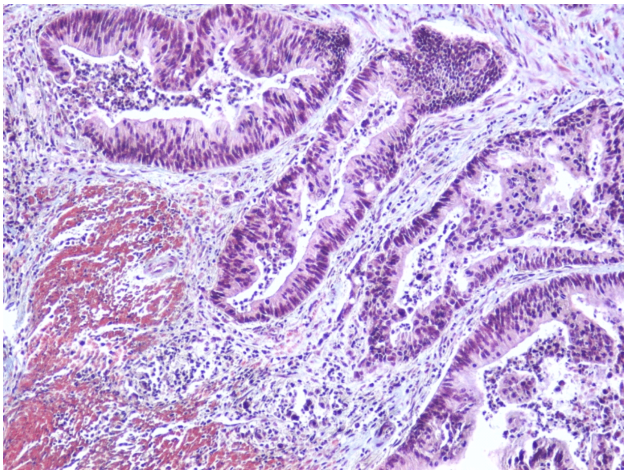

Figure 2 – ***Well-differentiated colon adenocarcinoma with intense inflammatory reaction. Trichromic GS staining, ×100.***

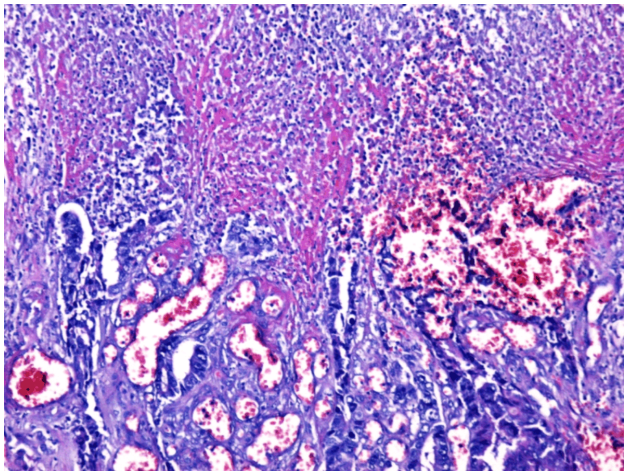

Figure 3 – ***Tumoral necrosis area developed in a moderately differentiated rectal adenocarcinoma with very intense inflammatory reaction. HE staining, ×100.***

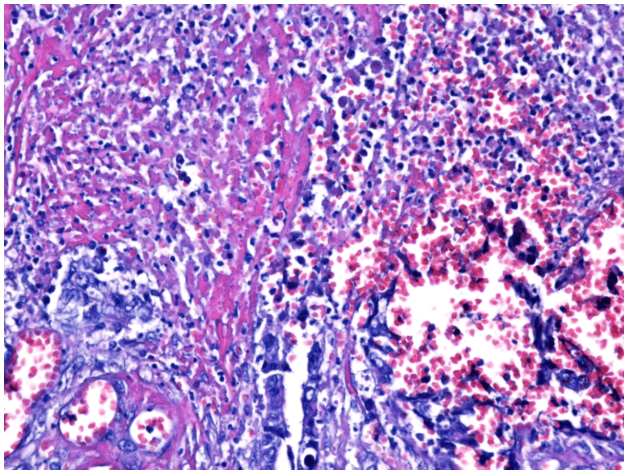

Figure 4 – ***Detail of the precedent figure showing the necrosis area with tumoral debris, vascular lesions with blood suffusions and inflammatory infiltrate rich in lymphocytes and macrophages. HE staining, ×200.***

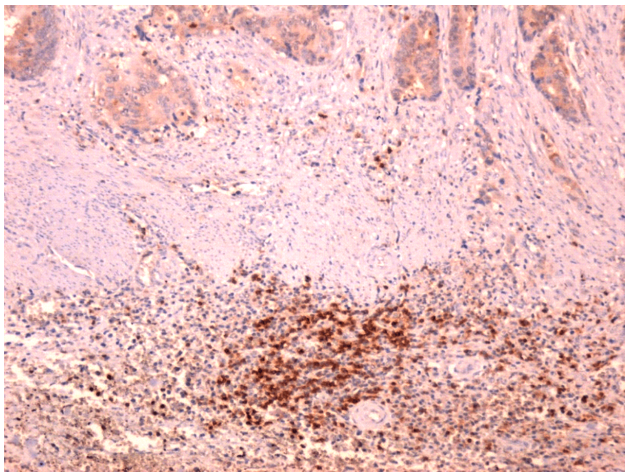

Figure 5 – ***Peritumoral stroma of colon neoplasm with an uneven distribution of T-lymphocytes. Anti-CD3 immunostaining, ×100.***

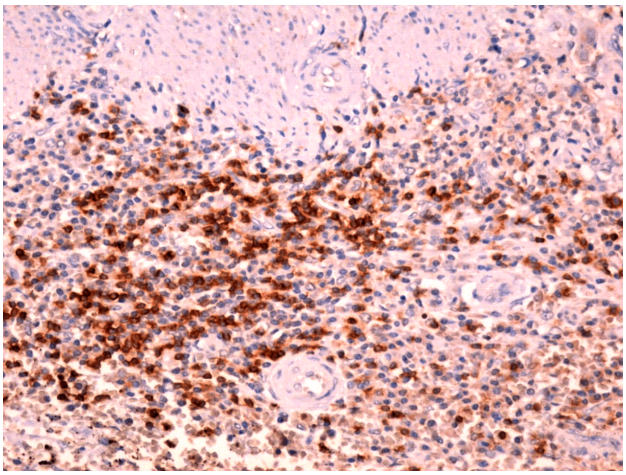

Figure 6 – ***Tumoral stroma with intense inflammatory infiltrate of T-lymphocytes (detail from the precedent figure). Anti-CD3 immunostaining, ×200.***

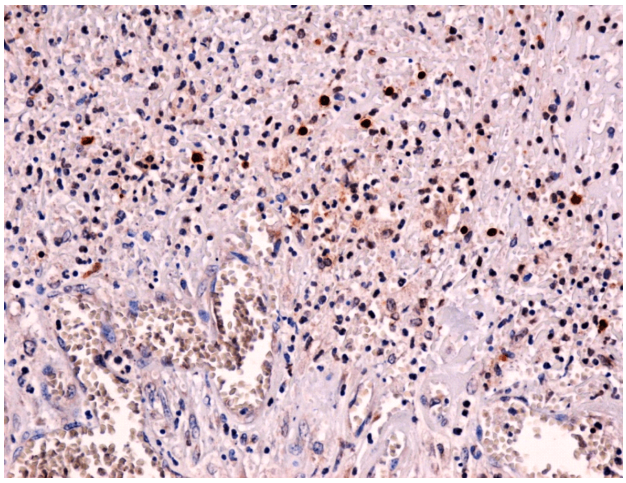

Figure 7 – ***Tumoral necrosis area with numerous, evenly distributed T-lymphocytes. Anti-CD3 immunostaining, ×200.***

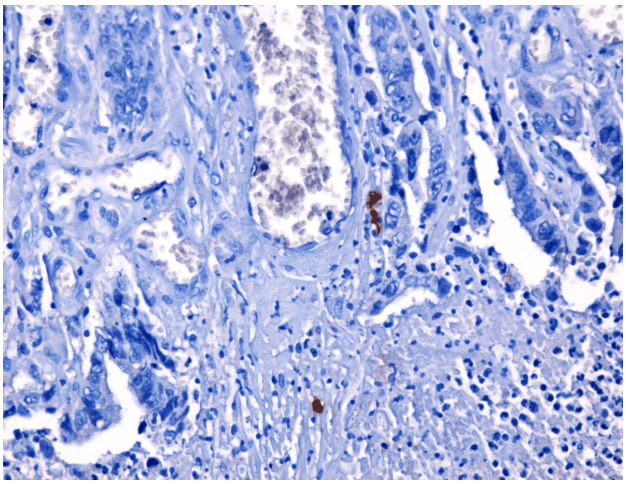

Figure 8 – ***Microscopic image of rare B-cells in the tumoral stroma of a moderate differentiated colon adenocarcinoma. Anti-CD20 immunostaining, ×200.***

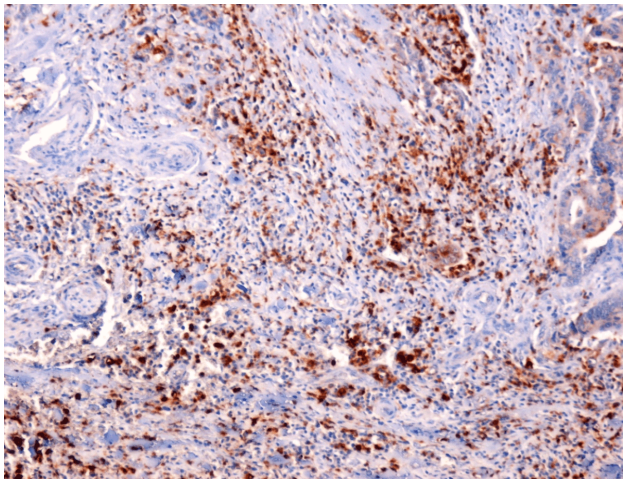

Figure 9 – ***Large population of macrophages in tumoral stroma. Anti-CD68 immunostaining, ×100.***

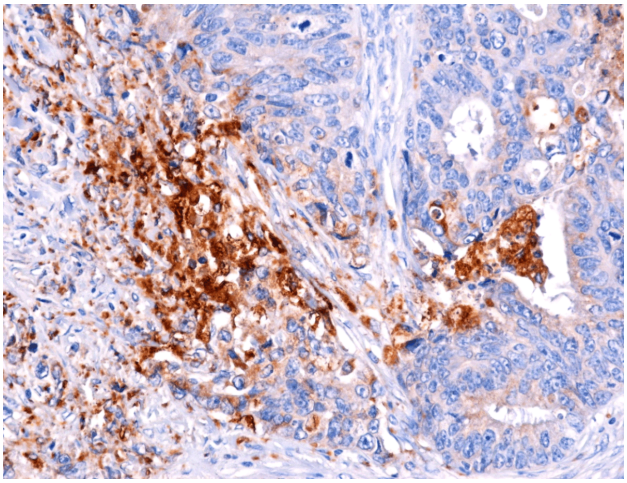

Figure 10 – ***Moderately differentiated adenocarcinoma with numerous macrophages disposed in the stroma but also intraglandulary and between tumor cells. Anti-CD68 immunostaining, ×200.***

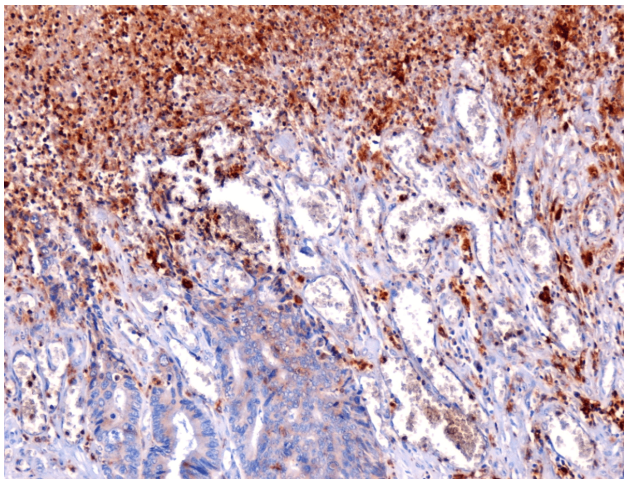

Figure 11 – ***Tumoral necrosis area highly infiltrated by the macrophages. Anti-CD68 immunostaining, ×100.***

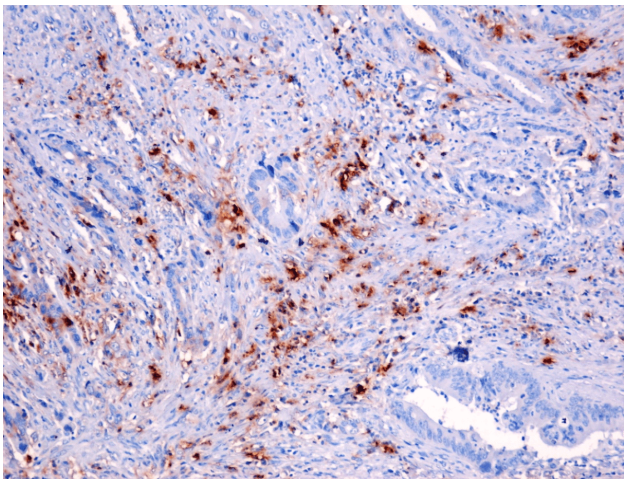

Figure 12 – ***Mast cells in the tumoral stroma of a moderately differentiated colon adenocarcinoma. Anti-tryptase immunostaining, ×100.***

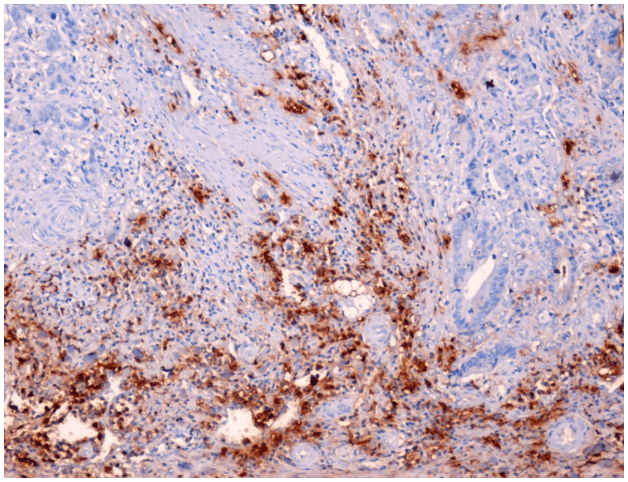

Figure 13 – ***Intense mast cells infiltrate in an area of the tumor. Anti-tryptase immunostaining, ×100.***

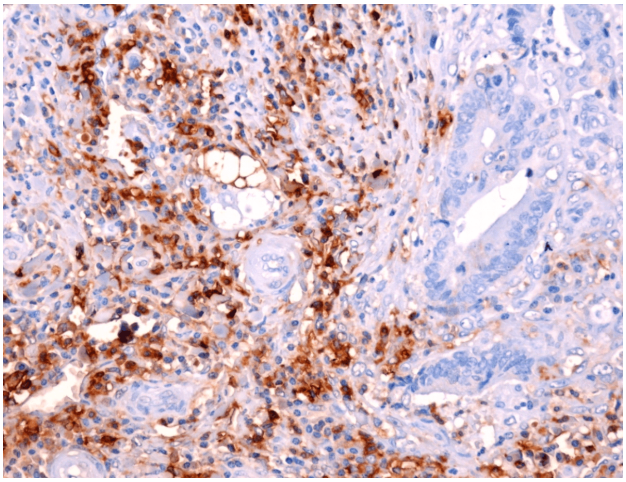

Figure 14 – ***Detail of the previous figure where we can notice the main perivascular disposal of the mast cells. Anti-tryptase immunostaining, ×200.***

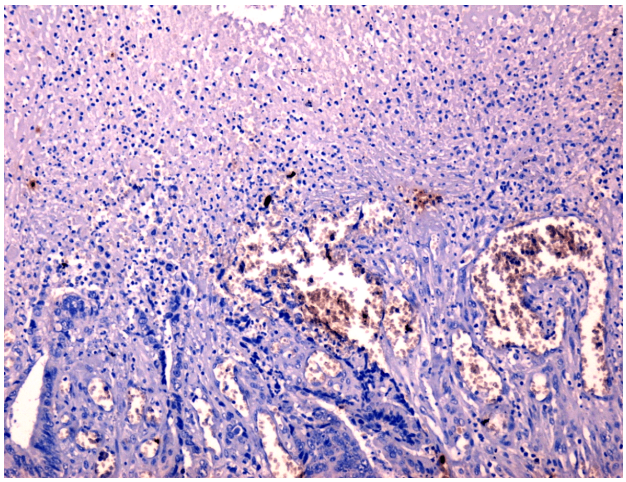

Figure 15 – ***Tumor necrosis area with absence of mast cells. Anti-tryptase immunostaining, ×100.***

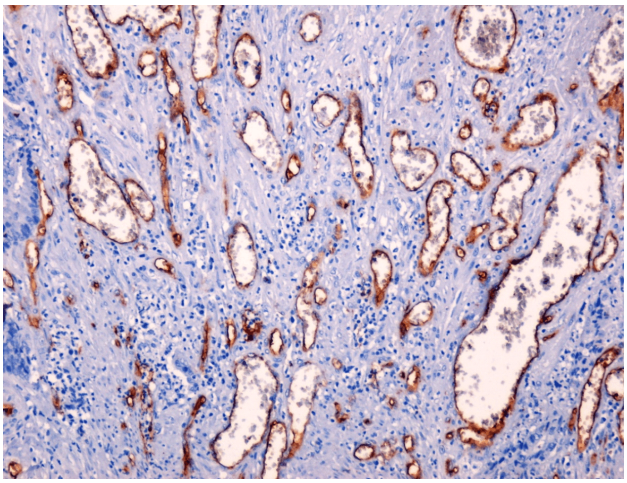

Figure 16 – ***Peritumoral stroma with abundant young vessels. Anti-CD34 immunostaining, ×100.***

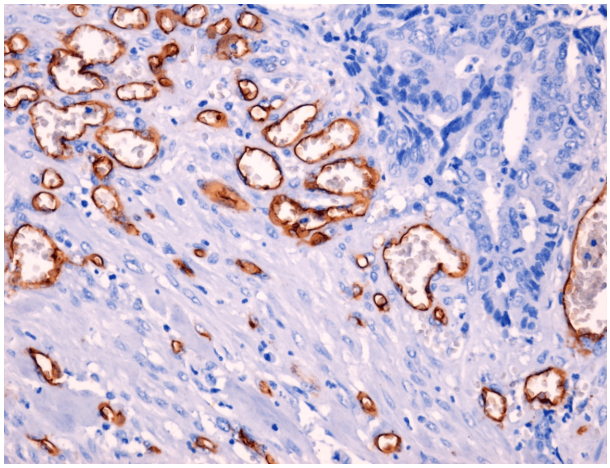

Figure 17 – ***Abundant vessels at the margin of a tumoral cluster. Notice the absence of inflammatory reaction. Anti-CD34 immunostaining, ×100.***

Discussion

Colon cancer is one of the frequent malignant conditions observed in clinical practice but also it is one of the important causes of death by neoplasm. Due to these reasons, the efforts for establishing the etiopathogenic mechanisms and for elaborating certain adequate therapeutic measures are highly motivated.

The relation between the neoplastic process and inflammation is incompletely established. According to some authors, the malignant condition and the inflammation have a strong relationship [12], but the intimate interaction mechanisms between the two processes are far from being completely understood [13]. If acute inflammation usually counter-balances cancer development, chronic inflammation promotes cancer development [7].

It is well-known the fact that inflammation is a physiological process that appears as a response to the intrusion of some antigens in the body by microbial infections, chemical irritations or traumas, which tend to alter homeostasis and produce tissular lesions [7]. When the inflammatory process has become a chronic one, there may produce changes in the cellular DNA, triggering the neoplastic process. The "key-players" of the inflammation-neoplasia relationship are the cells of the inborn immunitary system with their released secretion products [14].

The chronic inflammatory process is dominated by the presence of lymphocytes, plasmocytes, macrophages and mastocytes at the inflammation site. These cells

cooperate among themselves through the cytokines until the complete remake of the affected tissular structures. According to some studies, in cancer this cellular response of the body may suppress tumor development, or, on the contrary, it may facilitate cancer development through some signaling ways [14]. Most researchers admit that the cells of the inborn immunitary system, such as neutrophils, lymphocytes, dendritic cells and macrophages, may be present in the stroma of solid tumors [15]. That is why, in the present study, we evaluated the presence of these cells in the tumoral and peritumoral stroma, in order to highlight their reaction and their participation in the tumoral microenvironment.

In our study, the B-lymphocytes were almost absent in the tumoral stroma; instead, the peritumoral inflammatory infiltrate contained T-lymphocytes in the tumoral and peritumoral inflammatory reaction.

The most numerous cells present in the tumoral stroma were the macrophages. These cells were identified in large number in the immediate proximity of tumoral cells, intratumorally and even glandularly. The presence of a large number of macrophages in the tumoral necrosis areas is explained by the presence of some cellular and tissular debris that exert an intense chemotactic response among these cells. It is well-known the fact that macrophages are the main cells of the microenvironment in chronic inflammation, irrespective of its tumoral or non-tumoral character [6, 16]. These cells, alongside their physiological role of phagocytosis, generate a high level of reactive species of oxygen and nitrogen, with damaging effects on the cellular DNA [17, 18]. Most often, these chemical compounds produce mutations of the cellular DNA, being susceptible of triggering the carcinogenesis process. Also, it is well-known the fact that macrophages and T-lymphocytes may release the tumoral necrosis factor-alpha (TNF-α) and the inhibiting factor of the macrophage migration that exacerbates the DNA deterioration [19]. We identified numerous macrophages in the peritumoral stroma, in the immediate proximity of the tumoral cells, which indicates a very close relationship between the two types of cells. Also, we identified numerous macrophages among the tumoral cells. These macrophages, called tumor-associated macrophages (TAM), suppress the anti-tumoral response of the immunitary system by releasing the interleukin-10 (IL-10) and prostaglandin E2 (PGE2) [20] and also facilitate tumor growth by releasing some angiogenic factors, such as the vascular endothelial growth factor (VEGF), endothelin-2 and the plasminogen-activating factor [19, 21–23].

Other cells present in large numbers and peritumoral stroma tumor that we have investigated were mast cells (MC). The participation of mast cell in the tumor microenvironment formation has been less investigated. Mast cells are progeny of CD34+ hematopoietic stem cells involved in hypersensitivity reactions [24]. In the last two decades a few studies proved that MC play an important role in innate and adaptive immunity, intervening mainly in inflammatory processes and angiogenesis [25], closely interlinked processes, and also related to the tumor development and progression [26–28].

In our study, the highest density of MC was observed around congested blood vessels present in the tumor stroma. According to some studies, MC synthesize pro-angiogenic factors during inflammatory reactions that firstly induce the formation of new vessels and then site migration of inflammatory cells, amplifying the inflammatory process [29]. It seems that the MC plays an essential role in tumor angiogenesis by tryptase release in the tumor [30], which causes endothelial cell proliferation, release of IL-6 and granulocyte-macrophage colony-stimulating factor (GMCSF), both of them acting as proangiogenic molecules [31]. In addition, it appears that tryptase degrade extracellular matrix components and activates matrix metalloproteinases thereby promoting local tumoral invasion and metastasis [11, 32].

Anti-CD34 immunostaining exposed a rich network of blood vessels in the tumoral stroma of colorectal adenocarcinomas, a network that ensures the plastic and energetic support for tumor growth with local invasion, facilitating reproduction and metastasis. The vascular network was placed in the immediate vicinity of tumor cells, sometimes away from any inflammatory infiltrate. In our opinion, those aspects demonstrate that the peritumoral angiogenesis initiation and development can be controlled by tumor cells through biochemical factors synthesized by themselves, the inflammatory cells (mast cells, granulocytes, macrophages) interfering in some cases with that process.

The relationship between inflammation and cancer is very complex. Some authors consider that chronic inflammation affects all phases of carcinogenesis, from induction of the genetic mutation up to tumor microenvironment change, sometimes suppressing the immune response against tumor cells [33]. Other studies show that cancer cells induce an inflammatory response, which can be observed even in the early stages of carcinogenesis.

It is clear that tumor cells and inflammatory cells come to cooperate and form a tumor microenvironment that facilitates the tumor development and metastasis.

We believe that inflammation-cancer relationship is far from being known, but in the future will be an important therapeutic target.

Conclusions

A variable intensity chronic inflammatory infiltrate is always present in the tumoral and peritumoral stroma of colorectal adenocarcinoma, independent from the degree of tumor differentiation or other histological features. Among the cells of the immune system, the best represented in the peritumoral inflammatory infiltrate were macrophages and mast cells, and the least represented were B-lymphocytes. We found the highest number of macrophages in tumor necrosis areas, while mast cells were absent in those areas but very well represented around blood vessels. Macrophages were identified in a large number in the stroma, but also migrated within tumor cell islands or in the lumen of tumoral glands. We can speculate that macrophages might be responsible for "immune tolerance" against cancer cells. The tumoral stroma of colon neoplasm has a well-developed vascular network, that do not always correlate with the intensity of the inflammatory infiltrate; hence, we deduce that cancer

cells are able to develop their own vascular network through synthesis and secretion of proangiogenic factors with or without inflammation interference.

Acknowledgments

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Author contribution

All authors have contributed equally to the present work.

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