Research Article

Assessment of Inflammatory Status of Patients with Advanced Colorectal Cancer in Eastern Algeria.

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ABSTRACT

Lately inflammation has been suggested to promote cancer progression. It is generally believed that inflammation undertakes after initiation of tumor in the sporadic cancer, but the exact mechanisms involved in tumor developments are poorly understood. In our study we aimed to evaluate for the first time the inflammation response within the stromal microenvironment of sporadic colorectal cancer (CRC) in Algerian patients. Fresh tumor tissues were obtained from 30 patients of sporadic CRC with advanced stage (III.IV) and Histopatological slides were retrieved from archives of pathology center. Samples were scored according to Klintrup and Jass classification for inflammation grade. We also assessed inflammatory mediators such us nitric oxide (NO) and myeloperoxidase (MPO) and malondialdehyde (MDA) response. The results were shown as mean and standard deviation and P <0.05 was regarded as significant. Our histological examination revealed that 40% of patients had a high grade of inflammation, while the inflammatory stroma was characterized by a predominance of lymphocytes (35%). and a significant increase in MPO activity (P < 0.01) in CRC tumor tissues (10, 57 ±3, 39 U/mg tissue) correlated with a significant increase of NO release (2-fold) (6.97± 084),we have also noticed a highly significant (P<0.01) increase of MDA formation in CRC tumor tissues (71. 39± 10.07nmol/g tissue). Collectively, the assessment of inflammatory reaction in tumor revealed that high-grade of inflammation reflects the extent of inflammatory cell infiltration namely neutrophils and lymphocytes correlated with increased MPO activity, NO and MDA levels are thought to promote tumor progression in sporadic CRC.

Keywords: Colorectal cancer, Inflammation status, Myeloperoxidase, Neutrophils, Nitric Oxide.

INTRODUCTION

The response of the body to a cancer has many parallels to inflammation. German pathologist Rudolf Virchow is the first who found inflammatory cells in tumors, 150 years ago[1]. Lately the human inflammatory response to tumors has been more frequently investigated[2].

Inflammation has been positively correlated with cancer for many years. It's been evident that the interaction between malignant and inflammatory cells within the tumor stroma influences the tumor promotion, progression and aggressiveness, resulting to tissue reorganization[3,4].

Infiltrates of immune cells present in human neoplastic tumors is a common event. Furthermore, it is thought that the inflammatory reactions within the tumor are one of immune responses against malignant tumor cells[5,6]. Thereby, leading to leuckocyte extra vasation towards the damaged site[7].

Neutrophils are the most abundant circulating white cells and are the most prominent component of the first-line mechanism of defense against infection. However, neutrophils have been ignored for long time by immuno-oncologists. Percuraly, it is generally thought that high level of intra tumoral granulocytes can lead to tumor progression and associate with poor prognosis[8].

It has been proposed that some cytoplasmic granule constituents of neutrophil contribute to tumor development, blood vessels growth and cancer cells dissemination[9,10]. Myeloperoxidase (MPO), a plentiful peroxidase enzyme that neutrophils produce and release marked by its powerful pro-oxidant and pro-inflammatory properties to exert their microbicidal activity[11,12].

The neutrophils produce nitric oxide (NO) in response to extra cellular stimuli. Nitric oxide can also cause lipid peroxidation by stimulating hydrogen peroxide (H_2O_2) production and other oxygen derived reactive species[13].

Lipid peroxidation is a complicated process that implicates the oxidation of fatty acids resulting in the production of lipid radicals, damaging membrane lipids and the formation of breakdown products like Malondialdehyde (MDA). These cytotoxic compounds cause cellular macromolecules modification (i.e. DNA adducts, protein oxidation and glyco-oxidation) thereby altering tissue organization. Once formed, these breakdown products and depending on a variety of

events may either reduce or enhance cytotoxic potential[14].

Whereas, it is widely known that inflammation undergo after tumor initiation in the sporadic cancer, but the exact mechanism related inflammation to sporadic cancer remain unclear It is well known that the chronic inflammation, inflammatory bowel disease (IBD), may lead to the development of cancer, after an average of 30 years of evolution. Therefore, to more understand the inflammatory conditions in invasive CRC, the present study investigated for the first time the inflammatory status within the stromal micro-environment of human colorectal cancer advanced stage (III,IV) in Algerian patients and the possible release of MDA, NO and MPO as inflammatory response.

MATERIALS AND METHODS

Specimen collection

Human colon cancer tissues (Adeno carcinoma type) and paired normal tissues (10 cm adjacent to tumor) were obtained from 30 patients in surgery service of the Dr.Benbadis Hospital, University Center of Constantine, Algeria. Cancer tissues and matched normal tissues directly frozen and stored at -80 until use.

All the patients had no familiar history of colorectal cancer or inflammatory bowel disease,

Also we have excluded patients that had received chemotherapy or radiotherapy before surgical resection for colon cancer or benefits from treatment with non-steroidal anti-inflammatory drugs (NSAIDS).

All patients received an informed consent forms for sample collection. This study was approved by the Medical Ethic Committee of Dr. Benbadis Hospital, University Center of Constantine, Algeria.

Examination of Tumor inflammatory cell infiltration

The estimation of inflammatory cell reaction in tumor tissue was performed with the examination of histo-pathlogical haematoxylin and eosin (H&E) slides.

30 tumors samples from colorectal cancer patients newly diagnosed with advanced stage (III, IV), were retrieved from the pathology archives, a minimum of three slides from the deepest area of tumor invasion were selected and scored according to Jass and Klintrup criteria for peritumoral[15,16].

From 30 cases (59 years old, 23 male and 7 female). Only 20 cases were evaluated for invading margin, the remain cases were excluded from the study.

Inflammatory cell response was estimated at the invasive margin of tumor tissues. The amounts of lymphoid and neutrophil cells were noticed;

Score 0: Absence of increased infiltration of inflammatory cells.

Score 1: Slight increase of inflammatory cells infiltration but no destruction zone of cancer cells.

Score 2: Formation of a band-like infiltrate of inflammatory cells, and some destruction zone.

Score 3: An abundant inflammatory cells, with many/numerous destruction zone of cancer cells.

Myeloperoxidase (MPO) activity

MPO Activity an index of neutrophil infiltration was determined by a dianisidine-H2O2. Samples were suspended in 1ml of 50 mM sodium phosphate buffer incorporating 0.5% hexadecyltrimethylammonium bromide (pH=6.0), After homogenization, samples were frozen and thawed three times and were then centrifuged (12,000 rpm for 15 min at 4°C), and supernatants were further diluted into the same phosphate buffer containing 0.167 mg/ml o-dianisidinedihydrochloride and 0.0005% of hydrogen peroxide. The change in absorbance was measured at 460 nm (ε: 11, 300 M−1 cm−1). Results were expressed as units of MPO/mg tissue, where by 1 unit of MPO was defined as the amount of enzyme degrading 1 nmol H2O2 per min at 25°C[17].

Measurement of nitric oxide (NO)

Production of NO was evaluated by measuring the level of nitrite (an indicator of NO) in the tumor and adjacent tumor tissue supernatants using Griess reagent. Briefly, 100 µL of tissue supernatants were mixed with 100 µl glycine buffer and 200 µL Griess reagent (0.1% N-(1-naphthyl) ethylene diamine dihydrochloride, 1% sulfanilamide, and 2.5% H3PO4). After incubation at room temperature for 15 min, the absorbance at 545 nm was measured .The concentration of nitrite in the sample was determined from a sodium nitrite (NaNO2) standard curve and was expressed as µM.[18]

Measurement of MDA

The assay for membrane lipid peroxidation expressed as Malondialdehyde was tested. Reaction mixture containing aliquots of tissue homogenate, TCA (25%), TBA (0.67%), and BHT (0.01%) were incubated in boiling water for 45 min and centrifuged (3000 rpm, 10 min). Absorbance of the supernatant was taken at 535 nm. Amount of MDA formed is calculated from standard curve prepared using 1, 1', 3, 3' tetramethoxy propane and the values expressed as nmoles per g tissue.[19]

Statistical analyses

All data were expressed as means ± standard deviation (SD), values of $P<0.05$ were regarded as significant.

RESULTS

Patient clinical features

Table1 showed the clinical features of the study groups correlated with their inflammation statue and infiltrate immune cell type in colorectal cancer advanced stage patients. subdivided patients (30); (23 male,7 female;

mean age of 59 years old [38-82 years]) have an adenocarcinoma with a grade 1 predominance; well differentiated (83.33%) with a predominance of the left side of the colon 46,66% comparing to the right colon (26,66%) and rectum (16,16%). 20 patients have been subjected for grade inflammatory evaluation, results revealed that 60% of patients had a low grade of inflammation, only 40% had a high grade of inflammation, the inflammatory stroma was characterized by a predominance of lymphocytes (35%), and equal division between polymorph infiltrate immune cells and a predominance of PNN (25%). Also we can observe that low grade was characterized by an abundant infiltrate of lymphocyte instead of the high grade which was characterized by abundant infiltrate of PNN.

Table 1: Clinical features of patients n= 30, TNM classification and inflammation grade.

Clinical features	n(%)
Age	58,9±10,9
Gender	
Male	23(76,66%)
Female	7(23,33%)
Tumor site	
Right colon	16 (53,33%)
Left colon	9 (30%)
Rectum	5 (16,67%)
Grade	
G1	25 (83,33%)
G2	3(10%)
G3	2(6,67%)
Stage	
III	12 (40%)
IV	16(53,33%)
Undetermined	2(6,67%)
Inflammation grade	
High grade	8
Low grade	12
##	10
Infiltrate cell type	
Absent	3
PNN+++	5
LYM+++	7
Polymorph	5
##	10

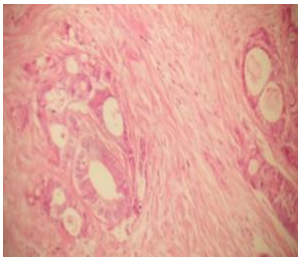
PNN+++: The infiltrate cells are characterized by the predominance of neutrophil cells,

Lym+++: The infiltrate cells are characterized by the predominance of lymphoid cells,

##: The estimation of inflammatory cell reaction at the tumor sections was impossible.

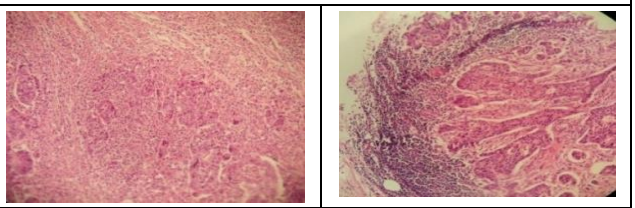
Tumor inflammatory cell infiltrate examination and MPO activity

Inflammatory cell reaction was estimated at the invasive margin of the tumor tissues. Invasive margin (20 cases) was defined as an interface between the host tissues and the invading edge area of a tumor. Micrograph of histopathological analysis using routine histology staining showed the areas of general inflammatory cell infiltrate. These areas are rich in inflammatory cells, including neutrophils, and lymphocytes which were recognized as small round cells (Figure 1, 2). Scoring analysis revealed that low grade indicated a mild increase of inflammatory cells, but no destruction of invading cancer cell islets (Figure 1.A, B), the high- grade indicated a very prominent inflammatory reaction, forming a cup-like zone at the invasive margin, with frequent destruction of cancer cell islets, characterized basically by PNN cell infiltrate. A central necrosis is often present in solid malignant tumor and is accompanied by an aggregation of leukocytes (Figure 2.A, B)



A B

Figure 1. Low-grade of tumor inflammatory cell infiltrate (S0: No inflammatory cell infiltrate) (A), (S1: Mild inflammatory cell infiltrate) (B), H& E,(x40).

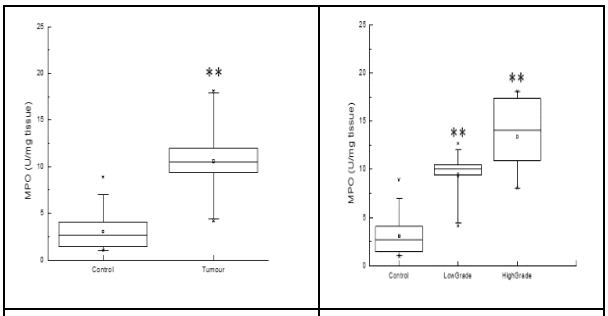


A B

Figure 2. High-grade of tumor inflammatory cell infiltrate (S2: increase of inflammatory cells with some destruction of cancer cell) (A), (S3: Severe inflammatory cell infiltrate, at the invasive margin, with frequent destruction of cancer cell islets) (B), H& E, (x40).

MPO level in tumor tissues

A highly significant ($P < 0.01$) increase was observed in MPO activity in CRC advanced stage (III, IV) tumor tissues (10,57 ±3,39 U/mg tissue) with respect to control tissues (3,058±1,84U/mg tissue) (Figure 3A). A severe increase was clearly mentioned in the high-grade samples (13,39 ±4,03 U/mg tissue) with respect to low- grade samples (9,44±2,50 U/mg tissue) (Figure 3 B). These results were clearly confirmed by the histological grade observation.

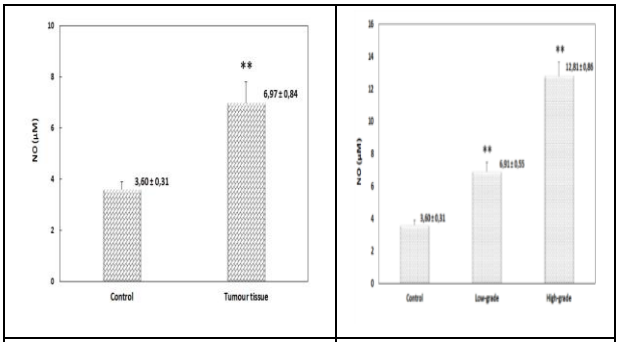


A B

Figure 3. MPO activity in the CRC tumor tissues (stage III ,IV) (Figure 3A); MPO activity in the High-grade and low-grade of CRC tumor tissues (Figure 3B). Values are mean ± SD, tumor tissues (n:30) vs control (n:30); High-grade sample(n:8) vs low-grade sample (n:13) **P<0.01.

NO level in tumor tissues

Figure 4A showed a significant (p <0.01) increase in NO level of tumor tissue (2-fold) (6.97± 084 uM) when compared to control values for adjacent tumor tissues (3.60±0.31 uM), the elevations in NO level was significantly (p < 0.01) more expressed (12.81±0.86uM) in high grade samples than low grade samples (6.91±0.55 Um) (Figure 4 B).



A B

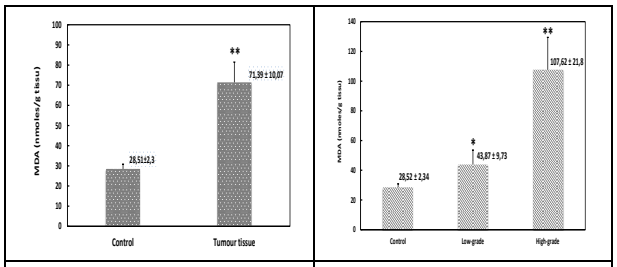
Figure 4.NO level in CRC tumor tissues (Figure 4A); NO level in the High- grade and low –grade(Figure 4B). Values are mean ± SD, tumor tissues(n:30) vs control (n:30); High-grade sample(n:8) vs low-grade sample (n:13) **P<0.01.

MDA Level in tumor tissues

A highly significant (P<0.01) increase in the MDA level was noticed in CRC tumor tissues (71. 39± 10.07nmol/g tissue)(Figure 5A). The MDA level in the in high grade-samples was found to be highly significant (P<0. 01) increase (107.62 ±21.8 nmol/g tissue) as compared to the low-grade samples (43.87±9.73 nmol/g tissue) (Figure 5B).

DISCUSSION

The present study aimed to define the inflammatory status within the stromal microenvironment of human colorectal cancer advanced stage (III,IV) and identified how this evolves during aggressive tumor in invasive stage.



A B

Figure 5.MDA level in CRC tumor tissues (Figure 5A); MDA level in the High- grade and low –grade(Figure 5B). Values are mean ± SD, tumor tissues(n:30) vs control (n:30); High- grade sample(n:8) vs low-grade sample (n:13) *P<0.05, **P<0.01.

In our investigation, the inflammatory cells which were abundantly infiltrated in tumor tissues namely neutrophils and lymphocytes were accompanied by central necrosis and an aggregation of leukocytes. In fact, findings on inflammatory status within the tumor sites are controversial. On one hand, diverse studies on how inflammation can foster tumor genesis have revealed that neutrophils play an important role in cancer development. Moreover, it has been found that neutrophils infiltration increases during the transition from normal to malignant mucosa. [20]

However, on the other hand, researchers have shown that the degree of tumor infiltration by inflammatory cells diminishes as CRC stage advances presumably representing a weak immune response versus cancerous cells. [21]

These data may justify our results which revealed more than 60% of tumors were accompanied by low-grade of inflammation. Surprisingly, it has been shown in a recent study that tumor infiltration with neutrophils have a positive prognostic impact among patients with CRC22,which may correlate with our observation that high-grade was characterized by the predominance of neutrophils in tumor infiltrating cells. Our study revealed that the destruction zones which were submerged and surrounded by neutrophil cells well correlate with increased MPO activity. In fact, a recent study suggests that MPO expressing neutrophils could exercise and promote direct anti-tumor properties on opsonized CRC cells[23,24]. Despite the presence of different immune cells associated with some immune responses in the tumor site, this event was unable to limit the colorectal carcinoma progression. From our funding, we suggest that the high inflammation in colorectal cancer advanced stage may be explained by the deleterious effect of some inflammatory mediators released during inflammation process such as RNS, MPO and MDA. It has been suggested that high levels of oxidative stress by products and amplified MPO activity may play a central role in CRC development.[25]

Our results revealed that MPO activity, MDA and NO levels were significantly increased in patients with high-grade of inflammation than those with low-grade. The

increased MPO activity well correlated with the extent of inflammatory cell infiltration observed by histological examination in the tumor tissues. In addition, there were areas of marked high-grade in the marginiamination tumor tissues. In fact, it has been reported that MPO reflects the presence of leucocytes in the inflammatory events and it is exploited as a marker of inflammatory syndromes.[25,26] Furthermore it has been reported that some neutrophils granule elements, such as MPO, may contribute to tumor genesis and cancer development 8. Neutrophils and in the presence of free radicals and MPO produces a potent oxidants to exert their effect. An excessive production of free radicals due to inflammatory responses might lead to DNA damage, proteins alteration and lipid peroxidation[27]. Therefore, cell integrity alteration and tumor formation[28, 29]. These data might explain our findings, since we have reported that MDA, a lipid peroxidation product, levels rise significantly in high grade inflammation of tumor tissue advanced stages in CRC.

Also we have reported a significant rise in NO levels in the tumor tissues as compared with controls. Moreover, we have noted a significant increase in high grade inflammation as compared with low grade group. NO might be generated by nitric oxide synthase (iNOS) that is believed to be over expressed in a large part of invasive CRC[30,31]. iNOS seems to take part in a range of human cancers. It has been reported that iNOS is highly activated in malignant breast cancer comparing to benign tumors or normal tissues[32]. Furthermore, the presence and levels of NO appears to affect the progression of primary tumors and metastasis events in breast cancer 33. High levels of NO have been suggested to promote mutagenesis and carcinogenesis in cervical, lung and gastric cancers 34, 35. In fact, NO has a multidimensional character in cancer. Interestingly, several studies have suggested that NO can both initiate and suppress tumor development and invasion[33]. Thus, its effect has been put into consideration depending on its timing, amount, exposure duration, emplacement and activity of NOS iso forms and levels of tissue exposure for long period especially at some point in chronic inflammation events which show an extended exposure to iNOS[36].

Furthermore, tumor microenvironment is marked by active inflammatory sites, accompanied mostly by stromal leucocytes, among them neutrophils and lymphocytes, and by hypoxic circumstances[27]. The rises ROS level during inflammation may be a result of the occurrence of leucocytes at the tumor site[37]. In fact, it has been demonstrated that nitrite ($NO2^-$), an NO metabolite, can be used by MPO to induce protein nitration and promote fatty acids peroxidation[38-40].

In conclusion, the present study defined for first time the inflammatory status within the stromal microenvironment of human colorectal cancer advanced stage (III, IV) in Algerian patients. Histological features reflect the extent of inflammatory cell infiltration, and tumor aggressiveness which clearly was associated with high levels of MPO activity, MDA, and NO amounts in high-grade of inflammation. Inflammatory cells in the tumor microenvironment may act as defenders influencing the tumor progression To more underling the molecular mechanism involved in tumors progression, further investigations on more inflammatory mediators in sporadic cancer needed for revealing new approaches to fight cancer development.

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