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Relationship Between the Levels of Oxidative Stress in Mesenteric and Peripheral Serum and Clinicopathological Variables in Colorectal Cancer

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ABSTRACT

Objective: To explore the differences existing between the levels of oxidative stress in peripheral and mesenteric serum in patients with colorectal cancer.

Material and Methods: One hundred fifty patients with colorectal cancer who underwent surgery between May 2005 and March 2010 were prospectively analyzed. The differences between oxidative stress parameters in their peripheral and mesenteric blood were measured. The associations between peripheral and mesenteric levels and the staging and clinicopathological variables were investigated.

Results: Oxidative stress parameters were higher in patients with advanced tumor staging ($p<0.01$), lymph node invasion ($p<0.01$), and venous invasion ($p<0.01$). Differences between oxidative stress parameters in peripheral and mesenteric blood samples were also observed.

Conclusions: The mesenteric levels of the oxidative stress markers were higher than the peripheral levels in these colorectal cancer patients. Higher levels of these oxidative stress markers are associated with an advanced state of cancer.

Key Words: Colorectal cancer, oxidative stress, reactive oxygen products

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Introduction

Colorectal cancer is one of the most frequently occurring types of cancer in humans and is one of the most frequent causes of death. There are a many pathological factors, including reactive oxygen species (ROS), involved in the process of cancer initiation and progression (1).

Free radicals are defined as molecules or molecular fragments with one or more unpaired electrons. Tissue damage caused by oxidative stress, mediated by excessive free radicals, is involved in a diversity of biological phenomena (2).

Lipid peroxidation can cause the destruction of cell membranes, thereby leading to cell death; early- and late-stage markers for lipid peroxidation include hexanoyllysine adduct (HEL), acrolein-lysine adduct (ACR), and 4-hydroxy nonenal (4-HNE) (3). Oxidative damage to DNA and RNA produces 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG), respectively, which are known markers of oxidative nucleoside damage (4). Damage to DNA, proteins, cell membranes, and mitochondria is involved in carcinogenesis, although no specific biochemical marker has yet been confirmed.

ROS are formed in excess in chronic diseases of the gastrointestinal tract (5), but the definite mechanisms of oxidative stress being induced in cancer cells and the role of ROS in colorectal cancer progression are still not entirely clear. Changes in some parameters of the anti-oxidative system in colorectal cancer were found in one study (6). The current research, comprised of a more extensive group of patients, is associated with the analysis of connections between the grade of lipid peroxidation as well as the parameters of the anti-oxidative system and selected clinical features of carcinoma.

The goal of this investigation is to explain the mesenteric and peripheral levels of malondialdehyde (MDA) and 4-HNE in patients with colorectal cancer and describe their correlation with staging and clinicopathological variables.

Material and Methods

The patients were volunteers and treated in accordance with the protocol approved by the research ethics committee of the local institution. In our study, 150 patients with colorectal cancer were prospectively analyzed. These patients were surgically treated by the General Surgery department, Haseki Education and Research Hospital, Istanbul and Yüzüncü Yıl University Faculty of Medicine, Van. The operations were per-

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formed between May 2005 and September 2010. Surgical resection was performed on 130 patients, while the tumors were considered unresectable in 20 patients.

Patients who had had some other benign or malignant neoplasia at some previous time, those for whom it was not possible to collect the data needed for the proposed analysis, and patients with chronic disease were not included in the study. The mean patient age was 58.5 years (21-80 years). With regard to gender, 59.4% of patients were female.

The variables analyzed included the staging of the colorectal cancer by means of TNM classification, the degree of cell differentiation, the diameter of the tumor, and the presence or absence of lymphatic invasion. According to the TNM classification, 35 patients were in Stage I, 40 were in Stage II, 42 were in Stage III, and 33 were in Stage IV. With regard to the degree of cell differentiation, there were 50 patients with well-differentiated (WD) tumors, 60 patients with moderately-differentiated (MD) tumors, and 40 patients with poorly-differentiated (PD) tumors. Regarding the diameter of the tumor, 41 patients had tumors ≤3.9 cm in diameter, 65 patients had tumors 4.0-7.9 cm in diameter, and 44 patients had tumors ≥8.0 cm in diameter. The presence of venous invasion was identified in the lesions of 37 patients, while lymphatic invasion was identified in 113 patients. Demographics and other selected characteristics of the cases are presented in Table 1.

Mesenteric blood samples were taken during surgery by a general surgeon. The blood was centrifuged, with the separation of the serum and plasma as well as the storage being performed in the same way as for the peripheral blood samples.

All samples were taken in the morning in order to avoid the confounding effect of diurnal variation of oxidative stress parameters, as reported previously (7). Ten mL samples of blood were collected in tubes containing lithium heparin, ethylenedinitrilotetraacetic acid (EDTA), or no additive, depending on the analysis. For protein oxidation parameters, plasma samples containing lithium heparin were stored at -80°C until analysis; some parameters were determined on the same day of collection (8).

Table 1. Demographics and other selected characteristics of the patients

Mean age (years)	58.5 (21-80)
Woman, n (%)	89 (59.4)
Tumor stage, n (%)*	
Stage I	35 (23.4)
Stage II	40 (26.6)
Stage III	42 (28)
Stage IV	33 (22)
Cell differentiated, n (%)	
Well	50 (33.4)
Moderately	60 (40)
Poorly	40 (26.6)
Tumor diameter (cm), n (%)	
≤3.9	41 (27.3)
4.0-7.9	65 (43.3)
≥8.0	44 (29.4)
Lymphatic invasion, n (%)	113 (75.3)
Venous invasion, n (%)	37 (24.7)

*Tumor stage was obtained according to the TNM classification criteria

TBARS Assay

The TBARS assay was prepared as described by Jentzsch et al. (9). In the TBARS assay, one molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) and thereby produces a pink pigment with an absorption peak at 535 nm. The amplification of peroxidation during the assay is prevented by the addition of the chain-breaking antioxidant, butyryl hidroxy toluene (BHT).

Plasma (400 µl) prepared by the hydrolysis of 1, 1, 3, 3-tetramethoxypropane (Sigma Chemical Co.) was mixed with 400 µl orthophosphoric acid (0.2 mol/L) (Sigma Chemical Co.) and 50 µl BHT (2 mmol/L) (Sigma Chemical Co.) in 12x72 mm tubes. A total of 50 µl TBA reagent (0.11 mol/L in 0.1 mol/L NaOH) (Fluka Chem.) was then added, and the contents were mixed. Subsequently, the contents were incubated at 90°C for 45 min in a water bath. The tubes were then kept on ice in order to prevent further reaction. TBARS were extracted once with 1000 µl n-butanol (Sigma Chemical Co.). The upper butanol phase was read at 535 nm and 572 nm in order to correct for baseline absorption in the Shimadzu UV-1601 (Shimadzu) UV-spectrophotometer. MDA equivalents (TBARS) were calculated using the difference in absorption at these two wavelengths, and quantification was performed with a calibration curve (10).

4-HNE Assay

4-HNE was measured by enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

Data are presented as means±SD. The comparison of the groups was performed using the Kruskal-Wallis one-way analysis of variance. $P<0.05$ was taken as significant. Binary (post hoc) comparisons and a Bonferroni-corrected Mann-Whitney U test (significance limit was taken as $p<0.0033$) were made. Analyses were performed using the SPSS 17.0 statistical package program. P values <0.05 were considered statistically significant.

Results

Two statistical analysis methods were performed, one numerical and the other categorical, and each of the markers was analyzed in relation to its peripheral and mesenteric concentrations. With regard to the numerical, descriptive measurements of the MDA levels, the mean for MDA (M) was 2.76 nmol/L±2.12 nmol/L and the mean for MDA (P) was 2.64 nmol/L±2.27 nmol/L, with a statistically significant difference ($p<0.05$). The comparison between the proportions of positive rates of mesenteric and peripheral MDA was performed

by means of a marginal homogeneity test. No statistical difference was found.

With regard to the numerical, descriptive measurements, the mean for 4-HNE (M) was 0.43 nmol/L±0.31 nmol/L and the mean for 4-HNE (P) was 0.38 nmol/L±0.25 nmol/L ($p<0.01$). To compare the evaluations of mesenteric and peripheral 4-HNE, a marginal homogeneity test was utilized, from which it was found that the rate of positive results was greater for mesenteric 4-HNE ($p<0.05$).

For both markers and for both mesenteric and peripheral blood, the levels were related to advanced stages of neoplasia, especially to Stage IV of TNM. In addition to this association, MDA (M) and MDA (P) presented correlations with venous invasion and lymph node invasion. These results are presented in Table 2.

Discussion

ROS are involved in a diversity of important phenomena in medicine, such as ischemia-reperfusion injury, pulmonary oxygen toxicity, atherosclerosis, mutagenesis, and carcinogenesis. The metabolism of ROS in cancer cells is a research area that has not been explored. Oxidative stress induces a cellular redox imbalance, which has been found in various cancer cells as compared with normal cells; the redox imbalance may thus be related to oncogenic stimulation. DNA mutation is an important step in carcinogenesis, and increased ROS levels have been established in various tumors. ROS can be involved in the initiation and promotion of carcinogenesis, the activation of proto-oncogenes, and the inactivation of stability and tumor-suppressing genes. They may oxidatively activate chemical carcinogenesis. Many studies have investigated most tumor markers, attempting to understand all the possible ways to use them in the diagnosis, staging, prognosis, and detection of tumor recurrences (11, 12).

The formation of ROS is a normal event in primal biochemical reactions. Oxygen radicals can be formed in elderly patients with chronic diseases of the gastrointestinal system (5). The primary source of oxidants in the gut is presumably phagocytes, which are accumulated in the mucus of patients with bowel diseases and could affect oxidants upon activation. This might contribute to the increased risk of cancer (13).

Oxygen radical production, which increases with the clinical progression of diseases, involves increased lipid peroxidation. Cellular membrane degeneration and DNA damage result from this. The extent of lipid peroxidation can be determined by estimating the final lipid peroxidation products MDA and 4-HNE, compounds known to produce protein cross-linking through Schiff's base, with DNA and DNA damage (14). Oxidative stress originates from an imbalance between the production of reactive oxygen/nitrogen species and the antioxidant capacities of cells and organs. ROS include superoxide anions (O_2.), hydroxyl radicals (.OH), and hydrogen peroxide (H_2O_2), while antioxidants are composed of several vitamins and endogenous enzymes, such as catalase, superoxide dismutase (SOD), and glutathione peroxidase. When the production of ROS exceeds the detoxification of ROS, the balance shifts towards oxidative stress. Oxidative stress to lipids, proteins, and nucleotides results in the accumulation of substrate-specific substances known as oxidative stress markers (2).

In colorectal cancers, the local cytokine network and the levels of nitric oxide (NO) and ROS are known to be closely related to cancer progression and metastasis (15). Similar to previous studies, we found that the levels of ROS in blood were higher in cases of advanced colorectal cancers. The levels of MDA and 4-HNE in colorectal cancer samples were significantly increased with the clinical staging of the disease. Our findings were in accordance with previous work that reported increased plasma MDA concentrations in colorectal cancer patients (16, 17). 4-HNE was found to be genotoxic in primary cultures of rat hepatocytes at low concentrations, which might occur in in vivo conditions of oxidative stress (18). However, coloncytes exposed to 4-hydroxy-2-nonenal in in vivo conditions could undergo a similar oxidative stress. Moreover, the reaction of aldehydes produced during lipid peroxidation with amino acid residues of proteins might lead to their oxidative

Table 2. Descriptive measurements of the oxidative stress markers and the histopathological variables and staging of the colorectal cancer (mean±SD)

Variables	MDA (M) nmol/L	MDA (P) nmol/L	4-HNE (M) nmol/L	4-HNE (P) nmol/L
Tumor stage*				
I	1.01±0.8	0.99±0.7	0.16±0.02	0.11±0.01
II	1.01±0.7	0.93±0.3	0.25±0.03	0.21±0.02
III	2.15±1.6	2.02±1.4	0.34±0.09	0.31±0.07
IV	3.99±1.9	3.28±1.8	0.41±0.03	0.38±0.02
p value	0.001	0.001	0.001	0.001
Diameter (cm)				
≤3.9	2.34±0.3	2.18±0.2	0.28±0.03	0.25±0.02
4.0-7.9	2.11±0.2	1.99 ±0.2	0.25±0.02	0.21±0.01
≥8.0	2.17±0.2	2.15±0.2	0.25±0.02	0.22±0.01
p value	0.107	0.188	0.104	0.105
Cell differentiation (D)				
WD	1.56±0.2	1.12±0.5	0.19±0.01	0.17±0.03
MD	3.67±1.4	3.28±1.1	0.31±0.02	0.28±0.02
PD	1.23±0.3	1.2±0.2	0.16±0.01	0.15±0.01
p value	0.814	0.631	0.243	0.198
Venous invasion				
Present	3.65±1.4	3.08±1.5	0.49±0.1	0.45±0.12
Absent	1.42±0.8	1.23±0.5	0.26±0.11	0.21±0.11
p value	0.036	0.021	0.169	0.091
Lymph invasion				
Present	3.71±0.3	3.12±0.7	0.45±0.02	0.41±0.03
Absent	1.67±0.4	1.24±0.4	0.26±0.03	0.21±0.02
p value	0.094	0.16	0.128	0.499

*According to the TNM classification criteria

modification (19). In this process, the final products of lipid peroxidation, such as MDA and 4-HNE as well as other products resulting from polyunsaturated fatty acid damage, could cause protein breakdown (20).

In our study, we showed significant differences between peripheral and mesenteric MDA levels. In the first investigation, the sample was composed of 250 patients who were analyzed retrospectively. All of them underwent the peripheral and mesenteric assaying of their oxidative stress marker levels, which were evaluated in relation to histopathological variables. Both of these markers had high levels in TNM Stage IV, both in mesenteric and peripheral blood. Thus, the markers had significantly higher levels when the neoplastic disease was no longer limited to the colon. These results probably indicate the presence of liver metastasis or occult lymph node metastasis. In our results, the mesenteric and peripheral oxidative stress levels were also higher in the presence of venous invasion. This may corroborate our hypothesis that drainage via the portal vein system is an important component of the distribution of these markers. These markers in the peripheral blood have still not been studied fully. They could be distributed via the portal vein system, the lymphatic systems, or both. Our results showed that there is a strong association between mesenteric and peripheral oxidative stress markers and the extent of venous invasion and the grade of invasion into the colorectal wall. These biomarkers have demonstrated usefulness in following up patients who have undergone surgery with curative intent, with increases in their levels in the event of potential tumor recurrence or the development of metastases. In this study, there were associations between peripheral and mesenteric oxidative stress levels.

Conclusion

These results demonstrated that, for the patients analyzed, there were significant differences between MDA and 4-HNE levels, with higher levels in the samples collected from the portal vein system than in those obtained from the peripheral blood. High mesenteric and peripheral reactive oxygen product levels were associated with venous invasion.

Conflict of Interest

No conflict of interest was declared by the authors.

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